American Journal of
Veterinary Research

CONTENTS

Experimental Infections of the Genital Tract of Swine and Goats with *Trichomonas Foetus* and Trichomonas Species from the Cecum or Feces of Swine 461
D. M. Hammond and W. Leidl

Swine Tissue Culture Systems. I. Trypsin-Dispersed Kidney 466
G. A. Young, N. R. Underdahl, and L. R. Sabina

Cytopathogenic Effects of Hog Cholera Virus on Embryonic Swine Tissues *in Vitro* .. 473
D. P. Gustafson and C. M. Pomerat

A Modified Assay for Vitamin B_{12} in Swine Blood Serum 481
G. L. Frederick

Blood Changes in Swine with African Swine Fever 484
D. E. DeTray and G. R. Scott

Shackling as a Factor in Ham Hip-Joint Hemorrhage 491
R. L. Kitchell, W. J. Aunan, L. W. Murphy, and D. P. Mossberg

The *in Vitro* Growth of Hog Cholera Virus in Cells of Peripheral Blood 502
H. W. Dunne, A. J. Luedke, C. V. Reich, and J. F. Hokanson

The Use of Low Level Piperazine on Pigs Naturally Infected with *Ascaris Lumbricoides* 508
W. D. Lindquist

Studies on the Pharmacology of Sulfaquinoxaline in Dairy Cattle 511
C. M. Stowe, D. Pallesen, and W. Hartman

The Metabolism of Phenothiazine, N.F., in Dairy Calves.
T. Ellison and A. C. Todd

I. Chronological Distribution of Drug in Digestive Tract Contents 519
II. Chronological Levels of Drug in Blood Serum, Bile, and Urine 522
III. Chronological Levels of Drug in Liver, Kidney, Spleen, and Mesenteric Lymph Nodes 526
IV. Chronological Total Drug Recovery 527

Infectious Bovine Rhinotracheitis 530
J. H. Gillespie, K. M. Lee, and J. A. Baker

The Cheek Teeth of Cattle 536
N. D. Jones and L. E. St. Clair

An Abnormality of the Bovine Penis 543
R. R. Ashdown

Methods of Injecting Tuberculin in Cattle 546
A. B. Larsen, L. A. Baisden, R. S. Merkal, and M. J. Morris

An Acidified Antigen for Detection of Nonspecific Reactions in the Plate-Agglutination Test for Bovine Brucellosis 550
J. E. Rose and M. H. Roepke

Interference Between Anaplasmosis and Eperythrozoonosis in Splenectomized Cattle 556
L. E. Foote, H. E. Levy, B. J. Torbert, and W. T. Oglesby

The Pathology of Virus Diarrhea of Cattle in Indiana 560
R. G. Carlson, W. R. Pritchard, and L. P. Doyle

The Endogenous Development of *Eimeria Zurnii*, a Pathogenic Coccidium of Cattle 569
L. R. Davis and G. W. Bowman

Isolation of *Vibrio Fetus* from Bulls 575
W. N. Plastridge, E. C. Walker, L. F. Williams, E. F. Stula, and E. M. Kiggins

(Continued on page 457)

A Publication of the American Veterinary Medical Association

Volume XVIII JULY, 1957 No. 68

Classified Advertisements Accepted in Research Journal

The *American Journal of Veterinary Research* accepts classified advertisements from graduate veterinarians seeking veterinary technological positions with colleges, universities, and other institutions such as industrial and commercial research laboratories.

Also, classified advertisements are accepted from institutions and other organizations which may be seeking veterinary bacteriologists, parasitologists, pathologists, or other veterinary technological personnel.

This service is intended to facilitate the placement or finding of personnel with specialized training in veterinary medical science.

The rate for these classified advertisements is $4.50 for the first 25 words and 15 cents for each additional word. No charge is made for use of a box number.

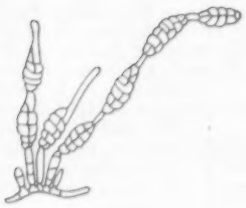
The closing date for classified advertisements in this *Journal* (a quarterly) is the 24th of the month preceding date of issue—for example June 24th for the July, 1957, issue.

(Contents continued from cover)

Observations on Experimental Bovine *Vibrio Fetus* Infection 579
H. Blobel, J. Simon, and S. H. McNutt
Parenteral Benzathine Penicillin V in Cattle 584
C. J. Hollister, R. A. Huebner, W. B. Boucher, and T. DeMott
Physiological Aspects of Fluids in Female Genitalia with Special Reference to Cattle—A Review 587
D. Olds and N. L. VanDemark
The Behavior of Spermatozoa in Luminal Fluids of Bovine Female Genitalia 603
D. Olds and N. L. VanDemark
Weight Gain in Phenothiazine-Treated Calves 608
G. E. Cauthen
Low Level Phenothiazine Administration to Weaned Beef Calves 612
G. E. Cauthen
Elementary Bodies of Sheep Pox 614
M. Abdussalam
The Cultivation of Sheep Pox Virus on the Chorioallantoic Membrane of the Developing Chicken Embryo 618
M. S. Sabban
Chlorinated Naphthalene Intoxication in Sheep 625
W. E. Brock, E. W. Jones, R. MacVicar, and L. S. Pope
Leukocytic Response in Lambs Exposed to Whole-Body X Irradiation 631
L. A. George, P. L. Hackett, and L. K. Bustad
An Electronic Stethoscope for Sterile Small Animal Surgery 634
J. A. Davis, B. D. Fremming, and H. M. Borella
Determination of Maltase Activity in the Blood Serum of the Dog 638
R. G. Skillen
The Use of a Bacterial Polysaccharide (Piromen) as a Pituitary-Adrenal Stimulant in Dogs 642
B. C. Wexler, J. L. Faehnrich, M. E. Weiss, and O. D. Grace
Symposium on Immunization Against Infectious Bronchitis Virus. I. Some Basic Properties of Infectious Bronchitis Virus 648
C. H. Cunningham
Pathogenic and Nonpathogenic Pleuropneumonia-like Organisms in Infectious Sinusitis of Turkeys 655
H. E. Adler and R. Yamamoto
Observations on a Chicken Embryo Lethal Orphan (CELO) Virus 657
V. J. Yates and D. E. Fry
A Comparison of the Protection-Inducing Factors in Chickens and Mice of a Type 1 Strain of *Pasteurella Multocida* 661
K. E. Yaw and J. C. Kakavas
Surgical Technique for the Exteriorization of the Ureters of the Chicken 665
J. M. Dixon and W. S. Wilkinson
"Atomized" Distemper Vaccine of Avian Origin. II. Field Experience in Mink 668
D. W. Johnson, V. J. Cabasso, K. Huffman, and M. R. Stebbins
Ringworm in Wild Animals in Southwestern Georgia 672
R. W. Menges, G. J. Love, W. W. Smith, and L. K. Georg
The Production and Use of Germ-Free Animals in Experimental Biology and Medicine 678
J. A. Reyniers
A Simple Ear Punch for Use on Laboratory Rats 687
B. J. Cohen and C. Chalberg
Heat-Inactivated Vesicular Stomatitis Virus as Antigen in the Complement-Fixation Test 688
G. T. Dimopoullos, O. N. Fellowes, J. J. Callis, G. C. Poppensiek, J. Tessler, and W. R. Hess
A Simple Technique for the Inoculation of the Chorioallantoic Membrane of Chicken Embryos 691
J. R. Gorham
Effect of Choline Deficiency on Chronic Selenium Poisoning of Rats 693
I. Rosenfeld and H. F. Eppson
A Turbidimetric Method for the Evaluation of Proteolytic Enzymes 698
S. W. Thompson, II
The Intradermal Sarcocystin Test 703
F. I. Awad
An Improved Method for the Rapid Cultivation of Large Yields of *Vibrio Fetus* 705
G. W. Robertstad and S. M. Morrison
Antigenic Differences Among Strains of *Leptospira Pomona* 708
A. D. Alexander, L. B. Evans, and R. Marchwicki

DIFCO

Media for



MYCOLOGY

DIAGNOSTIC

. . . for the isolation, identification and cultivation of pathogenic fungi. These media are also widely used in phytopathological studies. Several are neutral in reaction, giving optimum conditions for growth of a variety of fungi. The following may be prepared as selective media by the adjustment of reaction, addition of antibiotics or other agents:

Bacto-Brain Heart Infusion Agar
Bacto-Sabouraud Dextrose Agar
Bacto-Sabouraud Maltose Agar
Bacto-Littman Oxgall Agar
Bacto-Mycological Agar
Bacto-Mycological Broth
Bacto-Corn Meal Agar
Bacto-Corn Meal Agar with Dextrose
Bacto-Bean Pod Agar
Bacto-Prune Agar
Bacto-Lima Bean Agar

CONTROL

. . . for sanitary and sterility procedures as well as for general use in mycological procedures:

Bacto-Sabouraud Maltose Broth
Bacto-Sabouraud Liquid Medium
Bacto-Malt Extract
Bacto-Malt Agar
Bacto-Neurospora Culture Agar
Bacto-Potato Dextrose Agar
Bacto-Mildew Test Medium
Bacto-W.L. Nutrient Medium
Bacto-W.L. Differential Medium

CLASSIFICATION

. . . and nutritional studies of fungi:

Bacto-Yeast Morphology Agar
Bacto-Yeast Carbon Base
Bacto-Yeast Nitrogen Base
Bacto-Czapek Dox Broth
Bacto-Czapek Solution Agar
Bacto-Vitamin Free Yeast Base

THE DIFCO MANUAL, *NINTH EDITION,*
including descriptions of these media and their use,
is available on request.

DIFCO LABORATORIES
DETROIT 1, MICHIGAN

Editors

W. A. AITKEN, J. G. HARDENBERGH, HELEN S. BAYLESS

Committee on Journal: T. LLOYD JONES, WAYNE O. KESTER, W. W. ARMISTEAD.

Research Council

Anatomy and Histology

ROBERT GETTY, D.V.M., Ph.D., *(Chairman)*, Professor of Veterinary Anatomy and Histology, Division of Veterinary Medicine, Iowa State College, Ames, Iowa (1958).

Bacteriology (Immunology and Biological Therapy)

L. C. FERGUSON, D.V.M., Ph.D., Head, Department of Microbiology and Public Health, College of Veterinary Medicine, Michigan State University, East Lansing, Mich. (1959).

Biochemistry and Animal Nutrition

M. J. SWENSON, D.V.M., Ph.D., Department of Physiology, School of Veterinary Medicine, Colorado A. & M. College, Fort Collins, Colo. (1959).

Large Animal Medicine

S. J. ROBERTS, M.S., D.V.M., Professor, Department of Veterinary Medicine and Obstetrics, New York State Veterinary College, Cornell University, Ithaca, N. Y. (1959).

Large Animal Surgery

A. GORDAN DANKS, B.S., D.V.M., Professor, Department of Surgery, New York State Veterinary College, Cornell University, Ithaca, N. Y. (1957).

Parasitology

R. D. TURK, D.V.M., Head, Department of Veterinary Parasitology, School of Veterinary Medicine, Texas A. & M. College, College Station, Texas (1957).

Pathology

T. CARL JONES, B.S., D.V.M., (Lt. Col., V.C., U.S. Army), Armed Forces Institute of Pathology, Washington, D.C. (1959).

Physiology and Pharmacology

D. K. DETWEILER, V.M.D., M.S., Associate Professor of Pharmacology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pa. (1959).

Poultry Pathology

C. A. BRANDLY, D.V.M., M.S., Dean, College of Veterinary Medicine, University of Illinois, Urbana, Ill. (1957).

Radiology

MYRON A. THOM, B.S., D.V.M., Practitioner, Research Associate in Radiology, School of Medicine, University of Southern California, Los Angeles, Calif. (1957).

Small Animal Medicine

RICHARD L. OTT, B.S., D.V.M., Assistant Professor of Veterinary Medicine and Surgery, State College of Washington, Pullman, Wash. (1958).

Small Animal Surgery

JAMES ARCHIBALD, D.V.M., M.V.Sc., Professor, Head of the Division of Small Animal Medicine and Surgery, Ontario Veterinary College, Guelph, Ont. (1958).

Veterinary Hygiene

ALFRED G. KARLSON, D.V.M., Ph.D., Associate in Experimental Pathology, Mayo Foundation, Rochester, Minn. (1958).

Virology

C. H. CUNNINGHAM, D.V.M., Ph.D., *(Secretary)*, Professor of Microbiology and Public Health, College of Veterinary Medicine, Michigan State University, East Lansing, Mich. (1958).

Member-at-Large

BURTON J. GRAY, D.V.M., President, Fort Dodge Laboratories, Fort Dodge, Iowa (1957).

Published quarterly at 600 S. Michigan Ave., Chicago, Ill., by the American Veterinary Medical Association. Entered as Second-Class Matter January 31, 1941, at the Post Office at Chicago, Illinois, under the Act of March 3, 1879. Additional entry as Second-Class Matter at the Post Office at Champaign, Illinois, approved February 17, 1953.

Reproduction of any part of this publication is prohibited, unless special permission is given by the publishers and the authors.

Subscription price: $5.00 per year for United States, Canada and Pan American Countries, $5.50; foreign countries, $6.00. Members of the American Veterinary Medical Association may subscribe to the American Journal of Veterinary Research for $3.00 per year added to the annual dues of $15.00. Single copies $1.25. Address all correspondence to American Veterinary Medical Association.

The World's Standard Plant For The Investigation Of Rinderpest

EDWARDS CENTRIFUGAL FREEZE DRYING PLANT

— *No Shell or Pre-freezing!*

Keeping alive—without the loss of potency—vaccines and bacterial cultures is one of the most powerful aids to combating disease.

For this vital work EDWARDS FREEZE DRYING UNITS have been developed and are in use throughout the world making valuable contributions towards the mastering of RINDERPEST . . . NEWCASTLE DISEASE . . . BLUE TONGUE . . . BRUCELLA ABORTUS INFECTION . . . LUMPY SKIN . . . ETC.

Model 30 Series for Laboratory And Small-Scale Production

FREEZE DRYING CHAMBER

CAPACITY: Triple centrifuge plate (1200 0.5 cc ampoules) Bulk container (6 litres of solution). Alternative holders for 16 pre-frozen bottles, for vials, tray drying shelves, etc.

NEW VACUUM PUMP

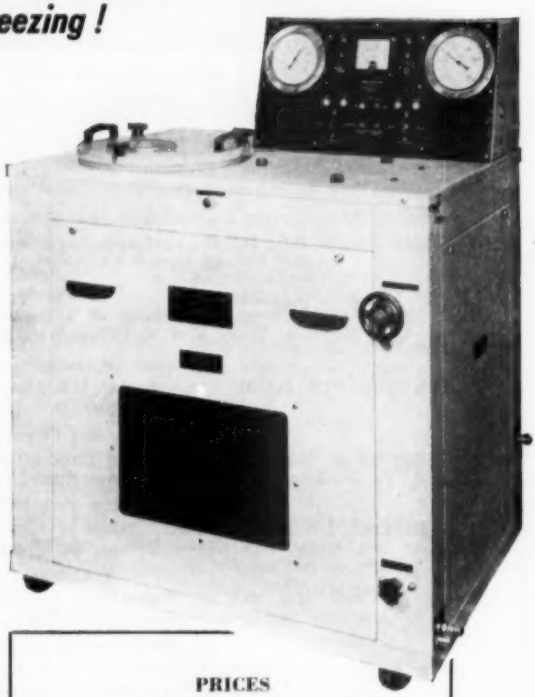
AIR-BALLAST MODEL fully protected against vapour contamination. New valve gives full protection against oil entering the systems.

WATER VAPOUR CONDENSER

Flood de-icing of the condenser chamber for quick de-frosting cycles.

SECONDARY DRYING

If necessary, secondary drying is carried out in a separate unit designed to operate with model above.



PRICES

For delivery C.I.F. New York

Model 30 Primary Dryer from $3,896
Model 30 Secondary Dryer from $1,098

VOLTAGES

110 volts 60 cycles or other standard supplies.

DIMENSIONS

Model 30 Primary Dryer 55" high x 45" wide x 30" deep.

EDWARDS HIGH VACUUM LTD.

MANOR ROYAL CRAWLEY SUSSEX ENGLAND

Phone: Crawley 1500 (10 lines) **Cables: Edcohivac, Crawley**

American Journal of Veterinary Research

Established 1940

COPYRIGHT 1957 BY AMERICAN VETERINARY MEDICAL ASSOCIATION

VOL. XVIII JULY, 1957 No. 68

Experimental Infections of the Genital Tract of Swine and Goats with *Trichomonas Foetus* and Trichomonas Species from the Cecum or Feces of Swine

DATUS M. HAMMOND, Ph.D., and W. LEIDL, D.V.M.

Munich, Germany

THERE is relatively little information about the host specificity of *Trichomonas foetus* and other trichomonads of domestic animals. *Trichomonas foetus* is able to produce infection in the vagina of sheep.[1] In other work, vaginal infections with *T. foetus* were not produced in sheep, swine, horses, goats, and cats.[2] Vaginal infections in cattle with trichomonads from the digestive tract of swine have also been reported.[3, 4]

This study was undertaken to determine whether *T. foetus* will produce infections in the genital tracts of swine and goats and to compare such infections in swine with those resulting from trichomonads from the digestive tract of swine. As yet, only one species of trichomonad from swine has been named. The name *Trichomonas suis* was given to the trichomonad from the stomach.[5, 6] There are two trichomonads in the cecum, a larger and a smaller form[7]; the larger form was used in this study. The relationship of this form to *T. suis* is yet to be determined but, in fresh material, it appears to be distinct morphologically from *T. suis*. After it has been maintained for some time in culture, the cecal trichomonad closely resembles *T. foetus* but, in fresh material, it is readily distinguishable from this species. The trichomonad from the feces of swine used in this study is presumably the same species as that from the cecum.

From the Gynecological and Ambulatory Veterinary Clinic of the University of Munich, Professor W. Baier, director.

During 1955–1956, the senior author was a Fulbright research scholar while on leave from the Department of Zoology, Utah State Agricultural College, Logan.

MATERIALS AND METHODS

The culture medium used for the trichomonads was prepared from peptone bouillon to which 0.1 ml. of horse serum and 1,000 μg. of streptomycin per milliliter were added just before use. After placing the medium in small tubes (3.0 ml. per tube) and inoculating, paraffin oil was added.

The strain of swine cecal trichomonads was isolated from cecal contents obtained from a pig in the municipal abattoir of Munich. Some difficulty was experienced in controlling the growth of bacteria and, after an experimental infection with the strain had been induced in the prepuce of a virgin bull known to be negative, a subculture from a preputial sample of this bull was used for further propagation of the strain. Swine fecal trichomonads were isolated from swine in the clinic. Vaginal samples were collected from swine with a plastic pipette and a syringe by introducing and withdrawing 20.0 ml. of physiological saline solution from the vicinity of the external os.

In the goats, 5.0 to 10.0 ml. of saline solution were introduced into the vagina and a portion of this was recovered in a beaker. Uniformly satisfactory vaginal samples were difficult to obtain from swine and goats. Preputial samples from goats were obtained by introducing 3.0 to 4.0 ml. of saline solution into the prepuce, massaging, and collecting the fluid in a beaker. Preputial samples from the boar were obtained by squeezing fluid from the prepuce or by a method similar to that used for goats.

Samples were usually collected twice weekly. After centrifuging, a portion of the sediment was examined under the microscope. The remaining portion was inoculated into three tubes of culture medium and these were incubated and examined three times at intervals of two days.

EXPERIMENTAL PROCEDURE AND RESULTS

Inoculations in Goats.—Three young virgin female goats were used. They were

TABLE 1—Results of Intravaginal Inoculations of Goats with Cultures of Trichomonas Foetus

No. of goat	Date of inoculation	Result of inoculation	No. of days infection demonstrable	No. of trichomonads in vaginal samples
1	4/ 9/56	+	29	Low
2	4/12/56	—	.....	.....
2	5/ 5/56	+	38	Low
3	2/24/56	+	22*	Low

* Goat killed 22 days after inoculation; trichomonads found in large numbers in uterus, cervix, and vagina.

in anestrus at the time of inoculation. Each goat was inoculated intravaginally with 2.0 to 3.0 ml. of *T. foetus* culture. The cultures originated from bovine preputial or vaginal samples except in one instance (second inoculation of goat 2) in which a culture was used which originated from a vaginal sample of goat 1. Two goats became infected as a result of the first inoculation, the third as a result of a second inoculation (table 1). Trichomonads could be found by direct microscopic examination in only one or two samples in each case; these occurred about midway in the period of infection in 2 goats and at the beginning of the period in the third. In the 2 goats in which the infection was allowed to run its course, samples were positive until 29 and 38 days, respectively, after inoculation. The third goat became emaciated and was killed 22 days after inoculation. Trichomonads were found in large numbers in the horns and body of the uterus, in the cervix, and in the vagina. Since vaginal samples had shown only relatively few trichomonads, the uterus apparently was the site of most active multiplication. No evidence of any pathological reaction to the presence of the infection was seen in any of the goats.

Preputial infections were attempted in 5 goats about 2 years old; 4 of these were in a state of sexual inactivity. Two goats received one inoculation, 1 received two, and 2 received three. The interval between successive inoculations of the same goat was about two weeks. In two series of inoculations involving 2 and 3 goats, respectively, a strain of *T. foetus* was used which had been recovered from a vaginal sample of 1 of the experimentally infected goats; in the other series of inoculations, cultures originating from bovine preputial samples were used. In each inoculation, 2.0 to 3.0 ml. of culture was injected into the preputial cavity, followed by light massage of the prepuce. No infection could be demonstrated following the inoculations in any of the goats. Four goats were sampled at various intervals from two to 48 hours after inoculation in three successive trials. All samples taken up to two hours after inoculation were positive, while all except one collected after six hours or more were negative; that one was positive after 24 hours. Therefore, *T. foetus* usually survived in demonstrable numbers for only a few hours after preputial inoculation. The preputial cavity of these goats normally contained less secretion than is usually observed in bulls. This may be a factor in the apparent inability of *T. foetus* to become established in the male goat.

Inoculations in Swine.—Five sows and 1 boar, 12 to 18 months old, were used in these experiments. Four sows were inoculated intravaginally with 3.0 to 6.0 ml. of *T. foetus* cultures. In each case, the sow was in heat at the time of inoculation and 3 of the 4 sows had been bred naturally or artificially just before inoculation. Two of the 4 sows experienced a demonstrable infection (table 2). Two sows were given a second inoculation 21 days after the first. In 1 of these (sow 2), a single sample, collected six days after inoculation, was positive by culture only. This may or may not represent a true infection. In the other 2 sows, definite infections occurred, which followed somewhat the same course. In 1 (sow 4), trichomonads could be seen di-

TABLE 2—Results of Intravaginal Inoculations of Swine with Trichomonas Foetus

No. of sow	Date of inoculation	Insemination	Results of examinations of vaginal samples taken at intervals of 2 to 4 days					
			1	2	3	4	5	6
2	10/14/55	Artificial	—	—	—	—	—	—
2	11/ 4/55	None	—	C	—	—	—	—
4	10/14/55	Natural	+	+	++	—	—	—
4	11/ 5/55	None	—	—	—	—	—	—
6	5/ 4/56	None	++	+++	++	—	C	—
7	1/30/56	Natural	—	—	—	—	—	—

C = positive by culture only; +, ++, and +++ = low, moderate, and high concentration of trichomonads, respectively.

rectly in low to moderate numbers in three samples collected four to eight days after inoculation. In the other (sow 6), trichomonads were found in moderate to high numbers in three samples collected four to 11 days after inoculation; one other sample, collected 20 days after inoculation, was positive by culture only.

Five sows were inoculated with 3.0 to 6.0 ml. of trichomonad culture originating from the digestive tract of swine (table 3). Four of the sows (2, 4, 6, 7) had previously received one or two inoculations with *T. foetus* and each except 1 (7) had become infected; the interval between the last inoculation with *T. foetus* and the first inoculation with swine trichomonads was 21, 20, 46, and 122 days, respectively. In 3 sows, cultures originating from swine cecal contents were used. In the other 2 sows (6 and 7), the culture originated from swine feces and the strain had undergone passage through the bovine male and female genital tract. The organism was recovered in vaginal samples from 4 of the 5 sows. However, in 3 sows, only the first sample, taken three or four days following inoculation, showed trichomonads in moderate numbers. In 2 sows, organisms were not demonstrated in any of the later vaginal samples. In 1 of these sows, reinoculation was followed by the finding of moderate numbers of trichomonads in the second vaginal sample.

The fourth sow (8) was bred naturally at the time of inoculation and experienced a normal pregnancy. The infection in this sow differed markedly from the others; it was more pronounced and of longer duration. Her first sample, collected four days after inoculation, was negative but, in the following seven samples, collected from the seventh to twenty-eighth day after inoculation, trichomonads were found in moderate to high concentrations. Four remaining samples, collected up to the forty-second day after inoculation, were positive by culture only or showed directly only a few trichomonads. This was the only sow inoculated with either species of trichomonad in which infection was associated with pregnancy; possibly the pregnancy influenced the infection.

One sow (6) showed an inflammation of the vulva the fourth day after inoculation with *T. foetus* and the vaginal sample collected at this time showed moderate numbers of trichomonads. However, the inflammation had disappeared by the fifth day, while the vaginal sample collected on the seventh day showed a high concentration of trichomonads. Therefore, it seems likely that the inflammation was due to causes other than the trichomonad infection. No other pathological reactions associated with any of the infections of either species of trichomonad were observed. Pregnancy did not result in any of the 3 sows bred at the time of inoculation with *T. foetus*. However, there is no evidence that the brief trichomonad infection of 2 of the 3 sows was a factor in failure to settle. As stated above, the 1 sow bred at the time of inoculation with trichomonads of swine origin conceived and farrowed normally in spite of a relatively severe trichomonad infection.

In comparing the infections caused by the two species of trichomonads, the small number of cases makes possible only tentative conclusions. The trichomonads of swine origin were found more consistently than *T. foetus* in the vagina of the sows following inoculations. However, there was more definite evidence of multiplication of *T. foetus* than of the trichomonads of swine origin, except in the infection associated with pregnancy.

Attempts were made to induce infections in a young boar. He was inoculated intrapreputially with 3.0 ml. of *T. foetus* culture and then about three weeks later

TABLE 3—Results of Intravaginal Inoculations of Swine with Trichomonas Sp. Originating from the Cecum or Feces of Swine

No. of sow	Date of inoculation	Insemination	Results of examinations of vaginal samples taken at intervals of 2 to 4 days					
			1	2	3	4	5	6
2	11/25/55	None	+++	—	—	—	—	—
4	11/25/55	None	++	C	C	C	—	—
6	6/19/56	None	—	—	—	—	—	—
7	6/ 5/56	None	++	—	—	—	....	....
7	6/19/56	None	—	++	C	—	....	....
8*	2/10/56	Natural	—	+++	+++	+++	+++	+++

C = positive by culture only; ++ and +++ = moderate and high concentration of trichomonads, respectively.

* The last positive vaginal sample was collected 42 days after inoculation; after this time samples were negative. This sow experienced a normal pregnancy.

was given a second inoculation with 9.0 ml. About a month after this, he received an inoculation with 6.0 ml. of culture of swine cecal trichomonads. No infection was found after any of these inoculations. It is likely that retention and decomposition of urine in the preputial cavity of the boar is a factor in making conditions unfavorable for trichomonads.

DISCUSSION

The results of these studies indicate that *T. foetus* is able to produce infections in the female but not in the male genital tract of goats and swine, and that trichomonads from the digestive tract of swine are able to become established in the vagina of sows. Since the female goats were in anestrus at the time of inoculation no information was obtained as to any influence of the infection on reproduction. There was no evidence that the infection with either species of trichomonad in swine has any effect on reproduction. This differs from the finding in heifers, by Fitzgerald *et al.*,[4] that infection with trichomonads of swine may be associated with infertility or abortion. However, the experiments with swine were too few to be conclusive. The course of infection with *T. foetus* in the female genital tract of goats differed from that which is typical in cows. Only small numbers of trichomonads were found in vaginal samples and the duration of infection was relatively short. However, in the goat which was killed during the infection, relatively large numbers of trichomonads were found in the uterus, which indicates that uterine infection may occur as readily in goats as in cattle.

The duration of infection in swine was usually even shorter than in goats. It may be questioned whether the trichomonads demonstrated in swine for only a few days represented true infections or merely survival for a time of the trichomonads inoculated. There was a more or less continuous gradation in duration from the shorter to the longer infections. In 1 sow only, the one in which infection of swine origin was associated with pregnancy, did the infection approach in duration that typical of *T. foetus* in cattle. This suggests that in the other sows the vaginal infection may have been curtailed by factors associated with the estrual cycle. The influence of the estrual cycle on *T. foetus* infection in cattle is known.[5] No information was obtained as to whether any penetration of the uterus by trichomonads occurred in swine.

The indicated lack of susceptibility of the male in both goats and swine provides an explanation as to why *T. foetus* apparently does not occur naturally in these species.

In work reported elswhere,[2] the authors have shown that trichomonads from the cecum or feces of swine are able to produce infections in the preputial cavity of bulls and that such infections can be transmitted by coitus to females and vice versa. These results, those reported in this paper, and the reports of experimental infections with *T. foetus* in the vagina of sheep [1] indicate that *T. foetus* and the trichomonad of swine origin have a relatively low degree of host specificity and that infections of abnormal hosts or organs with these trichomonads might be expected to occur occasionally under natural conditions.

SUMMARY

Low level vaginal infections in 2 goats resulting from inoculations with *Trichomonas foetus* cultures during anestrus were demonstrable for 29 and 38 days, respectively; numerous trichomonads were found in the uterus, cervix, and vagina of a third goat, killed 22 days after inoculation. No infections resulted from one to three intrapreputial inoculations with *T. foetus* in 5 goats.

Of 4 sows inoculated intravaginally with *T. foetus* cultures during estrus, 3 experienced infections demonstrable for three, eight, and 20 days, respectively. Four of 5 sows inoculated similarly with cultures of trichomonads from swine cecal contents or feces had infections demonstrable for three, four, seven, 12, and 42 days, respectively; the longest infection was associated with a normal pregnancy. A boar did not become infected following intrapreputial inoculations with either trichomonad species. No pathological changes attributable to trichomonads were observed in any of the experimental infections in swine and goats. The results indicate a relatively low degree of host specificity of these trichomonads.

References

[1] Andrews, Justin, and Rees, C. W.: Experimental *Trichomonas Foetus* Infection in Sheep. J. Parasitol., 22, (1936): 108.

[2] Trussell, R. E., and McNutt, S. H.: Animal Inocu-

lations with Pure Cultures of *Trichomonas Vaginalis* and *Trichomonas Foetus*. J. Infect. Dis., *69*, (1941): 18–28.

[2] Switzer, W. P.: Incidence, Cultivation, Morphology, and Certain Host Relationships of a Trichomonas Sp. (Protozoa: Zoomastigina) Occurring in the Nasal Cavity of Swine. M.S. thesis, Iowa State College Library, Ames, 1951.

[4] Fitzgerald, P. R., Johnson, A. E., Thorne, Joseph, and Hammond, D. M.: Experimental Infections of the Bovine Tract with Trichomonads from Swine. J. Parasitol., section 2 (Suppl.), *41*, (1955): 17.

[5] Davaine, C. J.: Traité des Entozoaires et des Maladies Vermineuses de l'Homme et des Animaux Domestique. 2nd ed. Bailliere, Paris, 1877.

[6] Gruby, D., and Delafond, H. M. O.: Recherches sur des animalcules se developmant en grand nombre dans l'estomac et dans les intestins pendant la digestion des animaux herbivores et carnivores. Compt. rend. Acad. Sci., *17*, (1843): 1304–1308.

[1] Buttrey, B. W.: A Morphological Description of a Tritrichomonas from the Nasal Cavity of Swine. J. Protozool., *3*, (1956): 8–13.

[3] Hammond, D. M., and Bartlett, D. E.: Pattern of Fluctuations in Numbers of *Trichomonas Foetus* Occurring in the Bovine Vagina During Initial Infections. Am. J. Vet. Res., *6*, (1945): 84–90.

[7] Hammond, D. M., and Leidl, W.: Experimentelle Infektion des Genitaltraktes beim Rind mit Trichomonaden aus dem Intestinum des Schweines. Berl. u. Münch. tierärztl. Wchnschr., *70*, (1957): 9–11.

Swine Tissue Culture Systems
I. Trypsin-Dispersed Kidney

GEORGE A. YOUNG, D.V.M.; NORMAN R. UNDERDAHL, M.S.;
LESLIE R. SABINA, M.S.

Lincoln, Nebraska

A SURVEY of the literature pertaining to the use of swine tissues in tissue culture systems disclosed that relatively little research had been conducted using tissues from this species. In much of the earlier work, swine tissues were used in an attempt to answer fundamental questions about origin and function of different cells.

Emmel [1] used 20- to 30-mm. swine embryos as a source of material in preparation of autoplastic cultures to study the origin of non-nucleated erythrocytes. The migration of epithelial cells was studied by Abromavich,[2] using tissues from swine placental areolae, the placenta, and the uterine wall at several different stages of gestation. Vinnikov [3, 4] used tissues from the eyes of swine embryos of different ages in an attempt to determine the origin of lens tissue. Galustian [5] grew epithelial cells from explants of embryonic pig thyroid but the follicle-like structures failed to produce colloid. The gallbladders from 6- to 25-cm. swine embryos were used in studies of epithelium by Grigoriev.[6]

Current interest is manifested in tissue culture systems as tools for virus research. Early work in this area was done by Hecke [7] in the cultivation of hog cholera virus in hanging drop cultures of embryonic choroid plexus, spleen, bone marrow, and lymph node. TenBroeck [8] also cultivated hog cholera *in vitro* on minced swine testicle but in a Maitland [9]-type culture rather than a true tissue culture. Boynton [10] also reported on the successful cultivation of this virus using bone marrow as the tissue source. More recently, Gustafson and Pomerat [11] reported cytopathogenic changes in tissue cultures from lung and spleen. Lee,[12] in 25 passages of hog cholera virus in swine kidney cell cultures, failed to observe cytopathogenic effects.

Other virus work with swine tissues includes use of lung and kidney tissues from day-old pigs by Wesslen and Lannek,[13] in studies of enzootic pneumonia of swine, and by Hjarre *et al.*,[14] in comparison of enzootic pneumonia to swine influenza virus. The use of swine embryonic tissues for cultivation of vesicular exanthema virus by McClain *et al.*[15] and Bankowski and Pfeifer [16] has been reported. Swine kidney cortex was used by Bachrach *et al.*[17] as host cells for foot-and-mouth virus and by Lee [18] to propagate transmissible gastroenteritis virus of swine.

In practically all the research with swine tissue cultures reported, the plasma clot technique was utilized. The work of Bachrach *et al.*[17] and of Lee [18] is excepted, as they utilized cells freed from the cortex of swine kidneys by trypsin.

The monolayer tissue culture systems obtainable by use of trypsin-dispersed cells has many advantages over tissue explants on plasma clots for experimental work with viruses. Because of this and the fact that trypsin-dispersed swine kidney cells are difficult to cultivate, the following investigations, on the development of procedures for the growth of these cells when dispersed by means of trypsin, were undertaken.

MATERIALS AND METHODS

Serums.—Serums from three species were used in these studies. Swine serums were used primarily and were from three age groups. A 20-liter pool of serum designated SWA (A for adult) was prepared from 1-liter aliquots of blood from 50 market hogs under CO_2 anesthesia. Blood from each hog was caught in a 2-liter porcelain cup and poured into 1-liter pyrex Roux bottles. The bottles were stoppered with paraffined corks and laid flat for four hours at room temperature during which time the blood clotted. They were then

From the Department of Animal Pathology and Hygiene, College of Agriculture, University of Nebraska, Lincoln. Published with the approval of the director as paper 797, journal series, Nebraska Agricultural Experiment Station.

This investigation was supported in part by research grant GR-4548 from the National Institutes of Health, U. S. Public Health Service.

The authors acknowledge the technical assistance of Sherrill L. Christiansen and express their appreciation to Dr. D. M. Pace for use of equipment and facilities of the Institute for Cellular Research.

Presented before the Section on Research, Ninety-Third Annual Meeting, American Veterinary Medical Association, San Antonio, Texas, Oct. 15–18, 1956.

stored upright at 4 C. overnight. The serum was decanted, centrifuged to remove red blood cells, and poured into a 5-gal. pyrex carboy. The serum pool was thoroughly mixed in the carboy, then distributed in 500-ml. aliquots to pyrex bottles, frozen, and stored at —20 C. This serum was not sterilized before storage but was filtered before use as described below for other serums.

A 600-ml. pool of serum was also made from blood of 12 pigs 4 weeks old which were obtained by hysterectomy and reared in isolation without receiving colostrum.[19] This serum was designated as SWY (Y for young). It was successively passed through sterile 015 and 0[3] Selas filters (*see* "Mediums," below), distributed aseptically to pyrex milk dilution bottles with Teflon-lined caps, and stored at —20 C.

A third pool of swine serum was obtained from the blood of 120 newborn pigs obtained by hysterectomy. This antibody-devoid serum was designated SWN (N for newborn) and was treated as SWY above.

Two bovine serums were also used in experiments to be described. Blood taken from a steer at slaughter yielded 3 liters of serum which was designated BOA. This serum was handled similarly to SWA. The second bovine serum, designated BON, was obtained by exsanguination of a calf soon after birth and before it had nursed. Serum was handled as SWY and SWN above.

One other serum used, designated as HOA, was obtained from the blood of an adult horse. Previous lots of serum obtained from the blood of this horse had been satisfactory for growth of Earle's strain L cells.[20] The serum was handled as SWY, SWN, and BON.

Tissues.—The kidney tissues used throughout these experiments were obtained aseptically from pigs 1 to 7 weeks old. All pigs were obtained by hysterectomy and were housed in isolation units on a modified cow's-milk diet until used.[21] They were anesthetized with chloroform and exsanguinated before removal of the kidneys. All instruments and glassware contacting the tissue were sterile and had been washed as described below. All manipulations of tissues were done under a hood or shield in a sterile room.

The kidney capsule was removed and slices of the kidney cortex approximately 5 mm. thick were obtained. They were transferred to a Petri plate, cut into small cubes of 3 to 5 mm. on a surface, and then transfrered to a 125-ml. modified Erlenmeyer flask (trypsinizer). Modification consisted of three horizontal indentations, a bowed-out bottom, and three small glass legs to accommodate the bowed bottom. These flasks equipped with a ¾-inch pyrex or Teflon-covered stirring bar served as efficient agitators when placed on a magnetic stirrer.

To the chopped kidney tissue in the trypsinizer were added 20 ml. of a 0.25 per cent trypsin (Bacto 1:250)solution in Hanks'[22] balanced salt solution (BSS) with added 1.4 per cent $NaHCO_3$ to obtain a pH of approximately 8.0. In general, the methods used were similar to those described by Youngner.[23] The tissue suspended in the trypsin solution was agitated at 37 C. for ten minutes with three subsequent decantations of trypsin and suspended debris.

Cells suspended in the fourth 20-ml. portion of added trypsin and successive cell suspensions were transferred to a 250-ml. flask containing 120 ml. of BSS with 10.0 per cent serum of 0.5 per cent lactalbumin hydrolysate (LA) at a pH near 7.2. This flask also contained a magnetic stirring bar and the suspended cells were rotated slowly to wash the trypsin from them. The flask was surrounded by ice water to hasten reduction of the trypsin activity. After four or five suspensions of cells were transferred in the washing flask, the suspension was poured into 90-ml. centrifuge tubes and the cells were spun down at 600 r.p.m. in an angle head of an International PR-1 refrigerated centrifuge. The cells were then resuspended in the washing diluent and recentrifuged. This procedure was repeated and then the cells were suspended in a basal medium for counting and inoculation into the varied growth mediums. They were used the same day or refrigerated overnight at 4 C.

Additives.—A number of materials were used in an attempt to stimulate growth of cells when added to basal mediums. For convenience, these are listed as additives.

Many of the additives were in the nature of tissue extracts. These were prepared by first mincing tissue with an equal portion of Hanks' BSS in a Waring blendor. They were then centrifuged at 1,000 x *g* for one hour. The supernatant was decanted, treated with hyaluronidase,[24] and centrifuged at 21,000 x *g* for one hour. The supernatant was filtered through an 03 Selas filter, poured in tubes in 6-ml. amounts, and stored at —20 C.

Preparation of extracts comprised two age groups of swine embryos, 30 to 40 days and 60 to 70 days. Because of possible toxicity, livers were removed from the latter group before they were minced.[25] Extracts were also prepared from 60- to 70-day-old embryos from liver, lung, and kidney and from kidneys of 30- to 40-day-old embryos and of 3-week-old pigs.

Other materials tested for growth-promoting activity in swine kidney cells were amnionic fluid surrounding 30- to 40- and 60- to 70-day-old embryos and bovine amnionic fluid of a 12-inch bovine fetus. The amnionic fluids were sterilized by filtration and stored in tubes at —20 C. Yeast extract (Difco) was also used. A 10 per cent suspension of yeast extract was first centrifuged and then the supernatant was passed through an 03 Selas filter, poured in tubes in 6-ml. amounts and stored at —20 C.

Mediums.—Two basal mediums were used primarily: (1) Hanks' BSS and 40 per cent swine serum SWA and (2) Hanks' BSS and 0.5 per cent lactalbumin enzymatic hydrolysate.[26] * Each of these basal mediums contained 100 units of peni-

* Nutritional Biochemicals Corp.

cillin and 0.1 mg. of streptomycin per milliliter. Control of pH was accomplished by variation of the volume of 1.4 per cent $NaHCO_3$ added and by aeration with CO_2. The phenol red in Hanks' BSS served as a satisfactory pH indicator. Merck's biological NaCl was used in BSS. Constituents of the mediums were sterilized separately either by autoclaving or filtration through a Selas 03 porcelain filter. The filtration system used was similar to that already described by Bryant *et al.*[28] Filtration was carried out under 10 lb. of pressure from compressed air containing 5.0 per cent CO_2. Complete mediums could be conveniently prepared with experimental variables by combining the desired sterile components which included the additives already described. All mediums were aerated with filtered 5 per cent CO_2 in compressed air before they were distributed to culture vessels.

Culture Vessels.—Culture vessels consisted of 12- by 75-mm. pyrex test tubes sealed with 00 silicone stoppers.† One milliliter of culture medium with suspended cells was added to these tubes. They were incubated at 37 C. in wooden racks at a 12-degree angle.

Culture tubes, glassware, and materials coming in contact with tissues or tissue culture (TC) fluids were cleaned with sodium hexametaphosphate (Calgon) and sodium metasilicate. The particular procedures used have already been described by Scherer.[27]

Enumeration of Cells.—Surviving and growing cells were quantitatively evaluated by liberating the cell nuclei with citric acid, staining the nuclei with crystal-violet dye, and counting them directly in a hemacytometer. The method described by Sanford *et al.*[26] served as the basis for the technique used. Citric acid was used at a 0.2 M, following the suggestion by Parker *et al.*[29] that higher concentrations of citric acid were more effective in freeing nuclei. Direct counts on trypsin-dispersed cells were made as a basis for dilution of cell suspensions before inoculation of tubes.

PROCEDURES AND RESULTS

Initial experiments to grow trypsin-dispersed swine kidney cells on pyrex glass were begun using swine serum SWA as the primary source of nutriments. This was based on the suggestion by Hanks[30] that dilute serum alone may suffice for the continuous production of several malignant cell strains.

Selection of a BSS and level of serum were also made early in the experimental program. A comparison was made between Hanks' BSS and Earle's BSS[31] in presence of 30.0, 40.0, 50.0, 60.0, and 70.0 per cent SWA serum with and without an embryonic extract (EE) which was added at a level of 2.5‡ per cent. The comparison of BSS was based on average nuclei counts of 45 tubes for each BSS. Hanks' BSS, including all levels of serum, held an advantage ratio of 1.17 in mediums without EE and an advantage ratio of 1.25 with EE present. Results of these comparisons were used as the basis to select 40.0 per cent of SWA serum as the most desirable level with Hanks' BSS.

Establishment of only 5.0 to 10.0 per cent of the trypsinized cells on glass, similar to that experienced by Youngner,[32] was also observed. An arbitrary inoculum of 1×10^6 cells per milliliter of medium was chosen. Results were similar with cells incubated statically and rotating ten cycles per hour. Use of static culture was selected because a larger number of tubes could be handled with the equipment available.

Tissue Extracts.—None of the tissue extracts used stimulated growth to a marked extent as determined by nuclear counts on seven, 14, and 21 days after inoculation of tubes with 1×10^6 trypsin-dispersed kidney cells. Embryonic extract from 30- to 40-day-old embryos essentially was nonstimulatory. The ratio of viable cells in mediums consisting of Hanks' plus 40.0 per cent serum (H + 40SWA) and the same medium plus EE was 1.0:0.9. It was not likely that failure of EE to stimulate growth was due to treatment with hyaluronidase, since untreated EE had a cell ratio of 1.0:1.0. Embryonic extract was used at 1.25, 2.5, 3.75, and 5.0 per cent levels. Results with EE from 70-day-old embryos were similar with a ratio of 1.0:0.8.

Results with extracts of lung (LE) from 60- to 70-day-old embryos were similar to those obtained with EE. Ratios were 1.0:1.1 for comparison of H + 40SWA and H + 40SWA + LE.

The other two extracts prepared from kidneys and livers of the same embryos showed evidence of inhibitory or toxic effects upon trypsin-dispersed kidney cells. Ratios to the basal medium were 1.0:0.4 for the kidney extract (KE) and 1:0.1 for the liver extract. Kidney extracts from 30- to 40-day-old embryos and from 3-week-old pigs were more favorable to growth of kidney cells. Both had ratios of

† West Co.

‡ Although an EE, 50.0 per cent, was used, 5 ml. was added per 100 ml. The 2.5 per cent is the corrected value for EE100. All figures for tissue extracts will be so presented.

1.0:1.2 on comparison to the H + 40SWA basal medium.

Growth Stimulants.—Several materials were incorporated into the TC mediums in an attempt to stimulate growth. Those used were trypsin, cobalt, yeast extract, and a lactalbumin enzymatic hydrolysate.

Trypsin (Difco) was added to a H + 40SWA + EE basal medium at level of 1, 10, and 100 mg. per milliliter of medium and incubated overnight at 37 C. These mediums were stored at 4 C. until used to inoculate tubes with trypsin-dispersed kidney cells. Levels above 1 mg. per milliliter caused changes in the mediums so that growth was unfavorably affected. Ratios to the basal medium were, respectively, 1.0:0.8, 1.0:0.2, and 1.0:0.1.

Cobalt nitrate was added to a H + 40SWA + EE basal medium as Co^{++} in concentrations of 0.1, 1.0, and 10.0 μg. per milliliter. The lesser concentrations of Co^{++} were neither stimulatory nor inhibitory, whereas the 10 μg. per milliliter level caused definite inhibition of cell growth (ratio, 1.0:0.03).

Yeast extract as an additive to a H + 40SWA basal did not promote more rapid or luxuriant growth of kidney tissues. The ratio was 1.0:0.8. Yeast extract levels of 0.05, 0.1, 0.25, and 0.5 per cent were used: the highest level was associated with severe granulation of cells by three weeks. Comparison of five mediums comprised of different serums with and without 0.1 per cent yeast extract disclosed a 1.0:0.9 advantage to the mediums in which yeast extract was absent.

Lactalbumin hydrolysate (LA) manifested a definite stimulatory effect on trypsin-dispersed kidney cells. The relative cell populations obtained are shown (table 1).

Addition of 0.5 per cent LA to a H + 40SWA basal medium markedly stimulated growth of trypsin-dispersed kidney cells as shown by the ratio of 1.0:8.0 on count of 21-day-old TC. Reduction of the serum to compare H + 40SWA to H + 5SWA + LA indicated that 5.0 per cent serum with 0.5 per cent LA present was a better growth medium for swine kidney cells than the H + 40SWA basal medium (ratio, 1.0:3.2).

TABLE 1—Relative Populations of Swine Kidney Cells in Mediums with and Without Lactalbumin Hydrolysate

	No. of cells x 10^6 at 21 days*		
	LA absent	LA present	
Experiment	H + 40SWA	H + 5SWA	H + 40SWA
1	25	58	203
2	20	89	165
Av.	23	74	184
Ratio	1	3.2	8.0

* Each figure represents average nuclear count of three tubes.

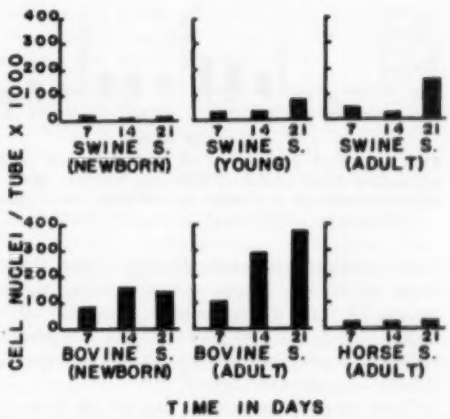


Fig. 1—Effect on growth of swine kidney cells by serums from different species of varied ages. Mediums consisted of addition of 5 per cent serum to a Hanks' lactalbumin basal medium.

Age of Tissue Donor.—Direct comparison of the influence of age of the tissue donor on cell population was not made. Tissues from several pigs of different ages should have been trypsinized and used to initiate TC on the same day but, because of limitation of technical help and facilities, this was not done. An indirect comparison was possible, however, with cell populations derived from different-age host tissues grown in an H + 5SWA + LA medium in different experiments. Tissues were obtained from pigs from 1 day through 7 weeks old. The cell population resulting from inoculation of 1 x 10^6 cells per tube was essentially the same regardless of age of the tissue donor. Fewer cells for inoculation were obtainable from the younger tissue donors.

Age and Species of Serum Donor.—Trypsinized swine kidney cells suspended in a H + LA basal medium were diluted to 1 x 10^6 per milliliter in mediums H + 5SWN, H + 5SWY + LA, H + 5SWA + LA, H + 5BON + LA, H + 5BOA + LA, and H + 5HOA + LA. Tubes were seeded with

Fig. 2—Effect on growth of bovine kidney cells by serums from adults of different species. Mediums consisted of a Hanks' lactalbumin basal medium with addition of 5 per cent serum.

these suspensions immediately after their final dilution. Nuclei counts were made seven, 14, and 21 days after seeding. Results based on nuclear counts on three tubes at each interval in replicate experiments are shown (fig. 1).

Newborn swine serum (SWN) at a level of 5.0 per cent in a H+LA basal medium failed to support significant growth of trypsinized swine kidney cells. Results with serum from 4-week-old pigs (SWY) were not much better. Adult swine serum (SWA) did support growth as did newborn bovine (BON) and adult bovine (BOA) serums. Growth was better with both of the bovine serums than with the adult swine serum. Adult horse serum (HOA) at a 5.0 per cent level was not satisfactory in an H+LA basal medium for growth of swine kidney cells.

An evaluation of the effect of the species of serum donor on the growth of trypsinized bovine kidney cells was also made. The conditions of the experiments were the same as those with swine cells. Results are given (fig. 2).

Good growth was obtained with mediums containing bovine serum and fair growth was obtained with mediums containing swine serum. Results with horse serum were not satisfactory.

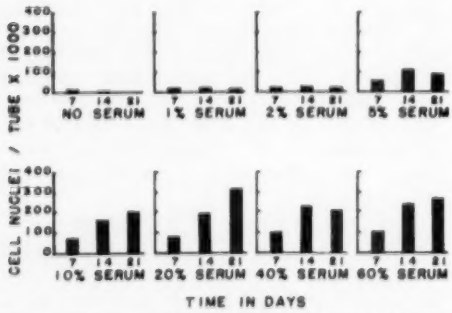


Fig. 3—Relative cell populations of swine kidney cells grown in mediums containing different concentrations of swine serum.

Serum Concentration.—The percentage of serum added to a H+LA basal medium which would stimulate good growth of swine kidney cells was determined for both swine and bovine serums in the same replicate experiments. In each of the three experiments, cells were prepared from the kidneys of 1 pig and suspended in a 1×10^7 concentration in a H+LA basal medium. Tenfold dilutions of these suspensions were made in each of the mediums containing 1.0, 2.0, 5.0, 10.0, 20.0, 40.0, and 60.0 per cent swine or bovine serum. A tenfold dilution of cells was also made in the H+LA basal medium.

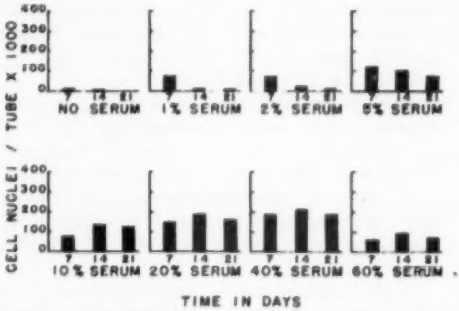


Fig. 4—Relative cell populations of swine kidney cells grown in mediums containing different concentrations of bovine serum.

Results obtained with different concentrations of swine serum are summarized (fig. 3). The H+LA basal medium was unable to support growth of swine kidney cells. The presence of small numbers of nuclei at seven and 14 days was due to cells which had adhered to the tube wall and had not autolyzed. Cells in mediums containing 1.0 and 2.0 per cent serum behaved similarly. There was some protective effect obtained from the presence of the serum. The medium containing 5.0 per cent serum supported growth of swine kidney cells although the cell populations obtained were relatively small. Good growth was obtained with mediums of 10.0 or more per cent swine serum. Optimum growth within these experiments was obtained with 20.0 per cent serum.

Swine kidney cells also grew with H+LA mediums containing different concentrations of bovine serum. Results of these ex-

periments (fig. 4) are similar in many respects to those obtained with swine serum. One obvious difference was the characteristic of small amounts of bovine serum, *i.e.*, 1.0 and 2.0 per cent, to have a more pronounced protective effect on trypsinized swine kidney cells than was given by swine serum. These concentrations of bovine serum were not capable of supporting growth of swine kidney cells. Mediums with 5.0 or more per cent bovine serum were capable of supporting growth. Best growth was obtained with 40.0 per cent bovine serum. Suppression of growth occurred in mediums containing 60.0 per cent serum.

DISCUSSION

There is a fundamental difference in the way trypsinized swine and bovine kidney cells grow. Cells from both sources begin growth as islets scattered over the lower surface of the culture tube as fixed for incubation. Bovine cells continue growth and the islets become confluent to form a continuous sheet of epithelial cells. These cells grow in such close proximity that moderate bulging of the sheet surface will occur in heavily populated tubes or flasks. This is characteristic even though kidneys of very young bovine fetuses are used as a source of cells. Although tissue cultures from young bovine fetuses are predominantly made up of fibroblasts, islets of epithelial cells do develop but become a part of a continuous sheet of cells on the glass surface.

It has been our experience that swine kidney cells do not form continuous sheets when grown on glass as is characteristic of bovine kidney cells. Even though a large population of cells is grown within a limited area, there are more or less distinct boundaries separating the islets of growing cells. Tissue cultures originating from swine kidney also contain a much larger proportion of fibroblasts than those originating from bovine kidney. Distinct epithelial islets among fibroblasts do not occur. Instead, fibroblasts and epithelial cells tend to be intermixed.

Our primary interest in tissue culture is the use of cells as hosts for the study of host-parasite relationships with swine viruses. For this type of research, absence of antibody in the growth and maintenance mediums is desirable. It was hoped that serum from newborn pigs which had not nursed would adequately support growth of swine kidney cells. Had this been so, this serum could have served as the source of nutriments for cells before and after addition of virus and no maintenance medium would be needed.

Use of serums from other species which may lack antibodies to the viruses being studied has a theoretical disadvantage. Viruses are intracellular parasites and their metabolism is often indistinguishable from that of the growing cell. Theoretically, highly specific viruses may need nutriments for their reproduction which are not available in serums of other species. The answer for current studies would seem to lie in use of serum from adults of the homologous species which have been managed in a manner precluding exposure to the virus under study. Eventual development and use of completely synthetic mediums which will support growth of cells and virus would be the ideal answer to the problem of antibody or inhibitory substances.

Cells grown from trypsinized swine kidney on glass consist of a mixture of cell types. Use of this type of tissue culture in study of animal viruses is justified by expediency. Considerable exploratory research can be conducted with such a system. Basic research on host-parasite relationships, however, had best be done on a quantitative basis which would involve use of pure cell lines. Numerical values for cells, virus particles, and antibody would place more authenticity on results obtained in host-parasite studies.

It is quite possible that so-called host specificity of many viruses is based on the cells which the viruses must attack in order to reproduce themselves. Inability of such viruses to establish themselves in foreign hosts may be due to absence of the specific cells they must attack or occurrence of those cells in such small numbers that inapparent infection occurs. Location and identification of such cells followed by their isolation and cultivation as pure cell lines would open new horizons in the study of host-parasite systems, particularly in the field of virus research.

SUMMARY

Growth of trypsinized swine kidney cells on glass in static tissue culture was

studied. Potential nutriments from a variety of sources were evaluated for their relative usefulness in growth mediums.

Swine embryonic extracts; extracts of fetal lung, liver, and kidney; and swine and bovine amnionic fluids did not increase cell growth when added to a basal medium comprised of Hanks' balanced salt solution and 40 per cent swine serum. Addition of Co^{++} or yeast extract or treatment of mediums with trypsin was also without effect. Definite stimulation of growth resulted from addition of an enzymatic hydrolysate of lactalbumin.

Evaluation of the influence of serums was made in a Hanks' lactalbumin basal medium. Serums from adult swine and cattle as a source of nutriments for swine kidney cells were preferred to serum from the young of those species, and also preferable to horse serum. Five per cent serum was found necessary for growth of cells but larger cell populations were obtained when greater amounts of serum were added to the basal medium. Optimum levels were 20 per cent swine serum and 40 per cent bovine serum.

References

[1] Emmel, V. E.: Concerning Certain Cytological Characteristics of the Erythroblasts in the Pig Embryo, and the Origin of Non-nucleated Erythrocytes by a Process of Cytoplasmic Constriction. *Am. J. Anat., 16,* (1914): 127–206.

[2] Abromavich, C. E.: Tissue Films from the Placenta of the Pig. *Anat. Rec., 44,* (1929–1930): 245–246.

[3] Vinnikov, I. A. A.: Growth and Transformation *in Vitro* of the Iridescent Portion of the Choroid Coat (Tapetum). *Akad. nauk SSSR Doklady (Acad. sci. URSS Compt. rend.), 20,* (1938): 211–213.

[4] Vinnikov, I. A. A.: Transformation and Proliferation of Elements of the Eye Lens in Tissue Cultures. *Akad. nauk SSSR Doklady (Acad. sci. URSS Compt. rend.), n.s. 52,* (1946): 725–728.

[5] Galustian, S. D.: *In Vitro* Cultivation of the Epithelium of the Thyroid Gland. *Akad. nauk SSSR Doklady (Acad. sci. URSS Compt. rend.), 23,* (1939): 937–938.

[6] Grigoriev, N. I.: Das Wachstum und die Verwandlung des Epithels der Gallenblase ausserhalb des Organismus. *Akad. nauk SSSR Doklady (Acad. sci. URSS Compt. rend.), n.s. 31,* (1941): 84–86.

[7] Hecke, F.: Die künstliche Vermehrung des Schweinepest-Virus mittels Gewekulturen. *Centralbt. f. Bakt., Abt., Orig. 1,* (1932): 517–526.

[8] Ten Broeck, C.: Cultivation of the Hog Cholera Virus. *J. Exptl. Med., 74,* (1941): 427–432.

[9] Maitland, H. B., and Maitland, M. C.: Cultivation of Vaccinia Virus Without Tissue Culture. *Lancet, 2,* (1928): 596–597.

[10] Boynton, W. H.: Preliminary Report on the Propagation of Hog Cholera Virus *in Vitro*. *Vet. Med., 41,* (1946): 346–347.

[11] Gustafson, D. P., and Pomerat, C. M.: Cytopathogenic Changes in Tissue Cultures Derived from a Pig Infected with Hog Cholera Virus. *Am. J. Vet. Res., 17,* (1956): 165–173.

[12] Lee, K. M.: The Application of Tissue Culture Methods to the Study of Virus Infections. *Rep. New York State Vet. Coll.* (1954–1955): 31.

[13] Wesslen, T., and Lannek, N.: The Isolation and Cultivation in Tissue Culture of a Cytopathogenic Agent from Pigs with Enzootic Pneumonia (So-Called Virus Pneumonia). *Nord. Vet.-med., 6,* (1954): 481–499.

[14] Hjarre, Von A., Dinter, Z., and Bakos, K. Über Züchtungsversuche mit dem Schweineinfluenzavirus (Shope) und dem Virus der enzootischen Schweinepneumonie *in vitro*. *Nord. Vet.-med., 6,* (1954): 919–935.

[15] McClain, M. E., Madin, S. H., and Andriese, P. C.: *In Vitro* Cultivation and Cytopathogenicity of Vesicular Exanthema Virus. *Proc. Soc. Exptl. Biol. and Med., 86,* (1954): 771–774.

[16] Bankowski, R. A., and Pfeifer, R. W.: Cultivation of Vesicular Exanthema Virus *in Vitro* Using Pig Embryo Tissues. *Proc. Soc. Exptl. Biol. and Med., 88,* (1955): 209–211.

[17] Bachrach, H. L., Hess, W. R., and Callis, J. J.: Foot-and-Mouth Disease Virus: Its Growth and Cytopathogenicity in Tissue Culture. *Science, 122,* (1955): 1269–1270.

[18] Lee, K. M.: Propagation of Transmissible Gastroenteritis Virus in Tissue Culture. *Ann. New York Acad. Sci., 66,* (1956): 191–195.

[19] Young, G. A., Underdahl, N. R., and Hinz, R. W.: Procurement of Baby Pigs by Hysterectomy. *Am. J. Vet. Res., 16,* (1955): 123–131.

[20] Sanford, K. K., Earle, W. R., and Likely, G. D.: The Growth *in Vitro* of Single Isolated Tissue Cells. *J. Nat. Cancer Inst., 9,* (1948–1949): 229–246.

[21] Young, G. A., and Underdahl, N. R.: Isolation Units for Growing Baby Pigs Without Colostrum. *Am. J. Vet. Res., 14,* (1953): 571–574.

[22] Hanks, J. H., and Wallace, R. E.: Relation of Oxygen and Temperature in the Preservation of Tissues by Refrigeration. *Proc. Soc. Exptl. Biol. and Med., 71,* (1949): 196–200.

[23] Youngner, J. S.: Monolayer Tissue Cultures. I. Preparation and Standardization of Suspensions of Trypsin-Dispersed Monkey Kidney Cells. *Proc. Soc. Exptl. Biol. and Med., 85,* (1954): 202–205.

[24] Bryant, J. C., Earle, W. R., and Peppers, E. V.: The Effect of Ultracentrifugation and Hyaluronidase on the Filterability of Chick-Embryo Extract for Tissue Culture. *J. Nat. Cancer Inst., 14,* (1953): 189–226.

[25] Hoffman, R. S., Fenebaum, E., and Doljanski, L.: The Growth Activating Effects of Adult Tissue on Fibroblast Colonies *in Vitro*. II. Experiments with Extracts of Brain, Bone Marrow, Liver, Lung, Smooth Muscle, Pancreas, Kidney, Ovary, Oviduct, Spleen, and Testis. *Growth, 4,* (1940): 207–221.

[26] Friedman, M., and Lieberman, M.: Propagation of Virus in Tissue Culture Employing a Simple Maintenance Medium Without Serum. *Fed. Proc., 15,* (1956): 587–588.

[27] Scherer, W. F.: An Introduction to Cell and Tissue Culture. (Chapt. 2, Glassware and Its Cleaning.) Burgess Publishing Co., Minneapolis, Minn., 1955.

[28] Sanford, K. K., Earle, W. R., Evans, V. J., Waltz, H. H., and Shannon, J. E.: The Measurement of Proliferation of Tissue Cultures by Enumeration of Cell Nuclei. *J. Nat. Cancer Inst., 11,* (1951): 773–795.

[29] Parker, R. C., Healy, G. M., and Fisher, D. C.: Nutrition of Animal Cells in Tissue Culture. VII. Use of Replicate Cell Cultures in the Evaluation of Synthetic Media. *Canad. J. Biochem. and Physiol., 32,* (1954): 306–318.

[30] Hanks, J. H.: An Introduction to Cell and Tissue Culture. (Chapt. 18, Nutrition of Cells *in Vitro*.) Burgess Publishing Co., Minneapolis, Minn., 1955.

[31] Earle, W. R.: Production of Malignancy *in Vitro*. IV. The Mouse Fibroblast Cultures and Changes Seen in the Living Cells. *J. Nat. Cancer Inst., 4,* (1943–1944): 165–212.

[32] Youngner, J. S.: Monolayer Tissue Cultures. III. Propagation of Poliomyelitis Viruses in Cultures of Trypsin-Dispersed Monkey Kidney Cells. *J. Immunol., 73,* (1954): 392–396.

Cytopathogenic Effects of Hog Cholera Virus on Embryonic Swine Tissues *in Vitro*

D. P. GUSTAFSON, D.V.M., Ph.D., and C. M. POMERAT, Ph.D.

West Lafayette, Indiana

RECENTLY, we reported[1] cytopathic changes in primary explant tissue cultures derived from a pig infected with hog cholera virus (HCV). The possible utility of such changes in diagnosis and research prompted this effort to find whether cytopathic changes would develop in primary explant cultures of embryo origin following *in vitro* exposure to HCV. Such findings would preface an effort to establish cell strains of swine origin which would support the growth of virus and show cytopathic changes due to its presence. The use of subculturable cell cultures would reduce or eliminate the objectionable features inherent in the use of primary explant cultures such as "wild viruses" and masking of cytopathogenic effects by antibodies.

MATERIALS AND METHODS

Swine Embryos.—Gravid uteri containing embryos 10 to 20 cm. long were selected at random, from a packinghouse,* without knowledge of the history of the sow.

Tissue Cultures.—Tissue cultures were prepared as previously described.[2] However, in the third trial, the medium consisted of 50 parts swine amniotic fluid, 45 parts Gey's balanced salt solution (BSS), and 5 parts EE50 (the supernatant fluid from a mixture of equal parts of ground 9-day-old chicken embryos and Gey's BSS), and 1,000 units of penicillin per milliliter. Tissue cultures prepared for five trials involved in the study were distributed as shown (table 1). The cultures in the first three trials were exposed to virus in 10 μg. of fluid by means of a micropipette. Exposures in the fourth and fifth trials were accomplished by placing 1.0 ml. of virus material or of control fluid with a 1.0 ml. of Gey's BSS in a watch glass containing the tissues prior to their being implanted in plasma clots. Cultures in the trials were exposed to HCV according to the outline (table 2).

Rabbit Spleen Extract.—This was supernatant fluid of a mixture of ground rabbit spleen and Gey's BSS—equal parts by volume. The spleen had been obtained from a normal rabbit and stored at —18 C. for five days.

Virus.—Three strains of virus (HCV) were used. A virulent strain (VV)† was used in amnionic fluid, urine, and whole blood from experimentally infected swine. The "rabbit modified" (RM)‡ virus and the virus "modified *in vitro*" ‖(MV) were used as received from commercial sources.

Hydrogen Ion Concentrations.—Hydrogen ion concentrations were approximated by color comparisons of tubes containing tissue cultures, using LaMotte phenol red standards.

Microscopy.—Scanning of cultures daily was accomplished with bright field conditions. Situations which seemed to require closer scrutiny were observed with phase microscopy. Also, photographic and cinematographic records were made under phase conditions.

Staining Techniques.—At various intervals, tissue cultures were fixed and stained in the technique suggested by Jacobson.[6] In addition, samples of control and exposed cultures of choroid plexus in the second and third trials were stained with Polak's stain for mitochondria.

RESULTS

Trials 1, 2, and 3.—The first three trials revealed no observable differences (with

TABLE 1—Number of Cultures of Each Tissue per Trial

Tissue	Trial number				
	1	2	3	4	5
Choroid plexus	.....	360	360	200	.....
Lymph node*	.....	.....	.....	200	800
Kidney	60	.....	.....	.....	.....
Cerebellum	60	.....	.....	.....	.....
Testes	60	.....	.....	.....	.....
Duodenum	120	.....	90	.....	.....
Spleen	120	372	360	200	.....
Heart	120	.....	.....	.....	.....
Liver	120	.....	.....	.....	.....
Lung	100	.....	.....	200	.....

* Lymph nodes cultured were prescapular, cervical, and prefemoral.

From the Department of Veterinary Science, Purdue University, West Lafayette, Ind. (Gustafson); and the Department of Anatomy, Tissue Culture Laboratory, University of Texas (Medical Branch), Galveston (Pomerat).

This work was supported in part by Office of Naval Research contract No. Nonr-1598(01).

Published as journal paper 1026 of Purdue University Agricultural Experiment Station, Lafayette, Ind.

* Houston Packing Co., Navigation Blvd., Houston, Texas.

† Virus obtained from the Hoosier Veterinary Laboratories, Thorntown, Ind.

‡ Virus obtained from Pitman Moore Co., Indianapolis, Ind.

‖ Virus produced by Cutter Laboratories, Berkeley, Calif.

TABLE 2—Number of Cultures Exposed to Hog Cholera Virus and Age of Cultures at the Time of Exposure

Tissue	Number of cultures/age of cultures in days at exposure				
	Trial number				
	1	2	3	4	5
Choroid plexus	.....	30/1, 30/2, 30/6, 30/8, 30/9.	60/1, 60/4.	100/0.*	.....
Lymph node	.....	.....	.....	100/0.*	400/0.†
Kidney	30/5	.....	.....	.....	.....
Cerebellum	30/5	.....	.....	.....	.....
Testes	30/5	.....	.....	.....	.....
Duodenum	30/5	.....	20/2, 20/3, 20/4.	.....	.....
Spleen	30/5	36/1, 36/2, 30/6.	60/1.	100/0.*	.....
Heart	30/5	.....	.....	.....	.....
Liver	30/5	.....	.....	.....	.....
Lung	30/5	.....	.....	100/0.*	.....

* Fifty were exposed each to "rabbit modified" virus (RM) and virus "modified *in vitro*" (MV).
† Exposed 100 each to RM, MV, and virulent virus (VV) in whole blood and in amnionic fluid. Controls were 100 each in amnionic fluid, rabbit spleen extract, and 200 were kept as environmental controls.

minor exceptions) between the cultures exposed to virus and the controls, either grossly, microscopically, or by difference in pH. On the ninth day of the second trial, choroid plexus cultures which were exposed to vv in amnionic fluid at six days showed a few focal areas of dead cells, increased cytoplasmic granularity and, in general, the outgrowths were less luxurious than those of the controls.

Also, in the second trial, 30 cultures of spleen and choroid plexus exposed to vv virus in whole blood 24 hours after culture were seriously impaired or inactive after another 24 hours. These cultures were apparently affected by the blood rather than the virus, since the material was used in undiluted form.

Cultures of the third trial were more erratic in their growth patterns in response to the medium, which was altered in this trial as described in "Materials and Methods." After four days, the spleen culture showed some outgrowth, but it was not nearly as luxurious as in the first two trials. At 16 days, only two of about 40 remaining cultures had viable outgrowths. Some of the choroid plexus cultures did show luxurious outgrowths while in others they were meager. Approximately the same was true of duodenum cultures which yielded epithelial outgrowth as opposed to wandering cells, fibroblasts, and epithelium of the choroid plexus; the epithelium was an outgrowth of the ependyma which is often attached to explants of the choroid plexus.

Polak's stain for mitochondria showed no clear difference in mitochondrial numbers or configuration between exposed or control cultures of choroid plexus in the second and third trials.

Trials 4 and 5.—In these trials, the explants were exposed to virus in watch glasses prior to their being embedded in plasma clots. In both of these trials, significant changes in the cytoplasm occurred in the cells of the outgrowths of the lymph node cultures. Lymphocytes were observed to die in 6-day-old lymph node cultures exposed to vv virus in amnionic fluid in

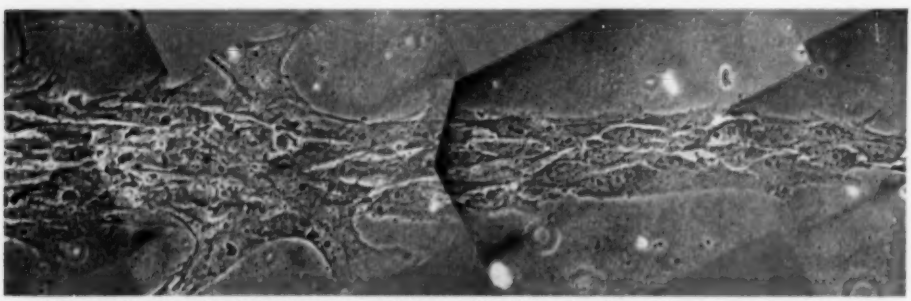


Fig. 1—Phase-contrast photographs of living cells. Normal culture of swine embryo lymph node showing fibroblasts arranged in a spikelike column which is considered to be endothelium in the process of fibroblastic transformation. x 200.

perfusion chamber experiments when cinematographic records were being made. Evidence of similar results was obtained in cultures exposed during the main portion of the fifth trial.

In the fourth trial, the lymph node cultures showed sprouts of fibroblasts and migration of wandering cells from control cultures as well as those exposed to RM and MV viruses on the second day. At three days, the control and MV-exposed cultures showed nice halos of fibroblastic reticulae which were clearly greater in amount than those of the RM exposure. On the fourth day, the control cultures showed many luxurious outgrowths of spikes of fibroblasts in transformations to endothelium which were based in the reticular sheet of fibroblasts. The culture cells appeared plump and glistening with homogenous cytoplasmic ground substance containing mitochondria and a normal apportionment of granules. Of the cultures exposed to RM virus, 36 per cent had fallen off the cover slips in the tubes and 72 per cent of the remaining cultures showed no outgrowths; of those that had outgrowths, some appeared to be like the controls. Of the cultures exposed to MV virus, 14 per cent had fallen off the cover slips and 20 per cent of those remaining showed no outgrowth, while 60 per cent of those which showed some outgrowth were markedly retarded in amount in comparison with the controls. On the fifth day, photographs were taken of endothelial cells in cultures of the controls (fig. 1, 2, 3) and those exposed to RM virus (fig. 4, 5, 6) and MV

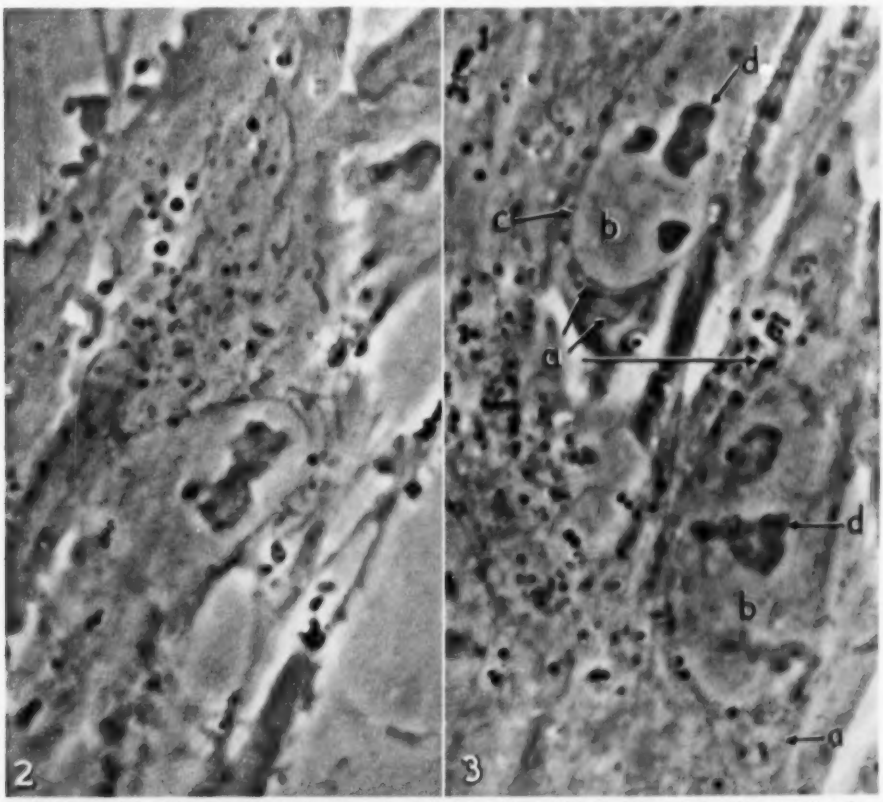


Fig. 2 and 3—Phase-contrast photograph of living cells. Cells of figure 1 showing details of cytoplasm of normal embryo lymph node outgrowth. Note the amount of cytoplasm and the filamentous mitochondria. Normal filamentous mitochondria (a), nucleus (b), nuclear membrane (c), and nucleolus (d). x 1,800.

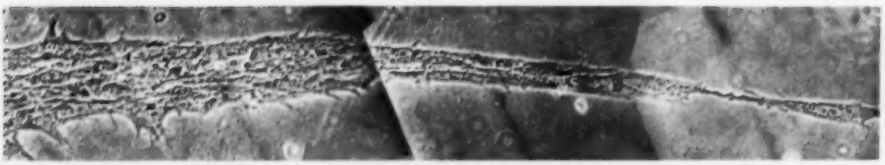


Fig. 4—Phase-contrast photographs of living cells. Culture of swine embryo lymph node exposed to "rabbit modified" virus. Note the spheroidal mitochondria with clear halos and the reduced cytoplasmic volume. x 171.

virus (fig. 7, 8, 9). In contrast to the cytoplasm of the controls, those exposed to RM and MV strains of virus showed large black granules circumscribed by clear light areas in the juxtanuclear zone of the cells, representing rounded or agglutinated mitochondria. Further, the cytoplasmic volume was reduced; this was evidenced by the filamentous strands of cytoplasm clinging to the cover slips following retraction of the cell membranes.

The fifth trial was designed to obtain more information pertinent to the results obtained in the fourth trial. The protocol for this trial and the distribution of fibroblastic sprouts among the 2-day-old cultures are shown (table 3).

The amount of outgrowth at five days (table 4) (ranging from none to minimal —indicating a few spicules of outgrowth; to fair—indicating more outgrowth about a considerable portion of the margin of the explant; to good—indicating a complete halo of outgrowth) was greatest in the control groups 6 and 7. The outgrowth of group 1 was the best of those exposed to virus; however, it fell considerably short of the development indicated in the control groups 6 and 7.

Cells of the outgrowths showed similar

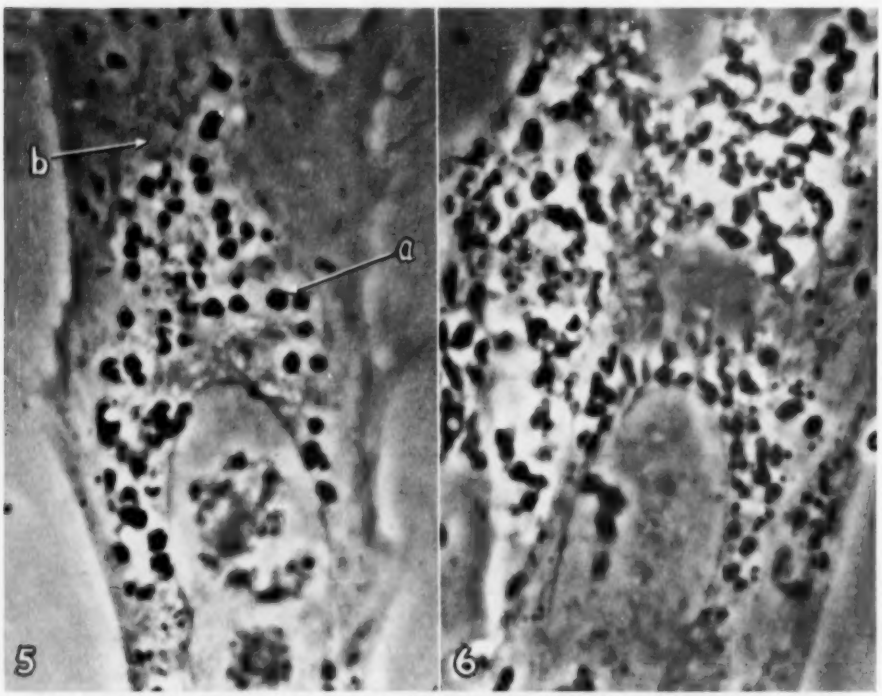


Fig. 5 and 6—Phase-contrast photograph of living cells. Cells of figure 4 showing details of the cells in contrast to those of figures 2 and 3. Pathological spheroidal mitochondrium (a) and normal filamentous mitochondrium with clear halo (b). x 1,800.

TABLE 3—Percentage of Explants Having Fibroblast Sprouts at Two Days Postculture

Groups		Per cent
1	100 exposed to RM virus	76.0
2	100 exposed to MV virus	64.0
3	100 exposed to VV virus in whole blood	28.0
4	100 exposed to VV virus in amnionic fluid	84.0
5	100 controls in amnionic fluid	21.0
6	100 controls in rabbit spleen extract	47.5
7	200 controls in nutrient medium (environment)	90.0

reactions to the presence of virus of all types, as described in lymph node cultures in the fourth trial, while none of the controls showed such cytoplasmic changes. The juxtanuclear zone of the cytoplasm of endothelial cells of spikes projecting from the reticular sheets of fibroblasts, and the fibroblasts, showed many spheroidal mitochondria with clear, light halos about them. The amount of cytoplasm was clearly less than in comparable cells of control cultures and showed evidence of having been reduced, as in the fourth trial.

Two additional cell changes that are believed to be important were detected by use of time-lapse cinematographic equipment and are best observed by this technique. Lymphocytes were observed to die in lymph node cultures exposed to VV virus. This was observed only by time-lapse cinematographic arrangements, although suggestive evidence is contained in table 4. The normal movement of lymphocytes seen in normal cultures gradually decreased in the exposed culture and stopped in approximately 12 hours. In this process, the lymphocytes became spherical and the cells appeared to be reduced to about one third of their normal size. The cell membranes of the endothelial cells bubbled, in a fashion quite like that observed in cell division, and the cytoplasm became reduced in volume. Following this, the cytoplasm appeared much more granular. Two cover slips bearing cultures were removed from a control tube and put under each of two motion picture units, whereupon one was perfused with undiluted swine amnionic fluid containing VV virus and the other was perfused with undiluted swine amnionic fluid. The experiment was repeated with the materials being diluted 1:1,000. The results were approximately the same in both efforts.

The cultures which were exposed to virus (table 4) appeared to show many more which had large numbers of "rounded-up" cells in a halo about them than did all three types of controls. These rounded-up cells are suggestive of the shape of dead lymphocytes. Likewise, those cultures exposed to virus showed a markedly higher number with extensive areas of liquefaction of the clot than did any of the three control groups. The percentage of explants which dropped off the slides was comparable among all groups, except for the amnionic fluid control group 5.

The pH of the cultures was read at two, five, and seven days postculture. With the exception of group 5, which was constantly higher than the other groups, there was no significant difference in pH between the groups (table 3).

The choroid plexus cultures of the fourth trial showed no difference between those exposed to either RM or MV virus until the third day. At this point, those exposed to RM virus showed much less outgrowth, ranging from explants with no outgrowth to some that were as luxurious as the controls in outgrowth. The MV virus-exposed cultures were similar to the controls. This situation remained constant through the fourth, fifth, and sixth days postculture.

During the periods of observation in the fourth trial, no observable differences were noticed between those cultures of spleen

TABLE 4—Distribution of State of Cultures at Five Days Postculture in Percentages*

Group	Amount of activity				Condition of culture		
	None	Minimal	Fair	Good	Explants loose	Rounded-up cells†	Liquefied clots‡
1	29	8	20	16	4	13	10
2	38	16	17	7	1	14	7
3	33	7	7	2	5	26	20
4	22	14	11	2	1	35	15
5	37	20	3	2	38	0	0
6	19	6	11	43	9	9	3
7	38	5	7	38	3	6	3

* Groups are the same as indicated in table 3.

† Aggregates of dead cells which were suggestive of lymphocytes; these were free cells away from the main outgrowth.

‡ Liquefied areas about the explants were quite extensive, at least one half of the perimeter.

Fig. 7—Phase-contrast photographs of living cells. Culture of swine embryo lymph node exposed to virus modified in vitro. Note the similarity of this culture to that of figure 4 and the contrast to that of figure 1. x 240.

or lung exposed to virus and their respective controls.

Subsequent trials to help confirm the results were conducted elsewhere. These trials involved over 500 explant cultures of swine embryo lymph nodes. The fibroblast-like cells of the outgrowths showed changes like those described in the fourth trial following exposure to HCV in each of the following vehicles: swine urine, amnionic fluid, and a commercially prepared immunizing agent. The strain of virus in the urine and amnionic fluid was VV; in the immunizing agent, it was MV. The control cultures

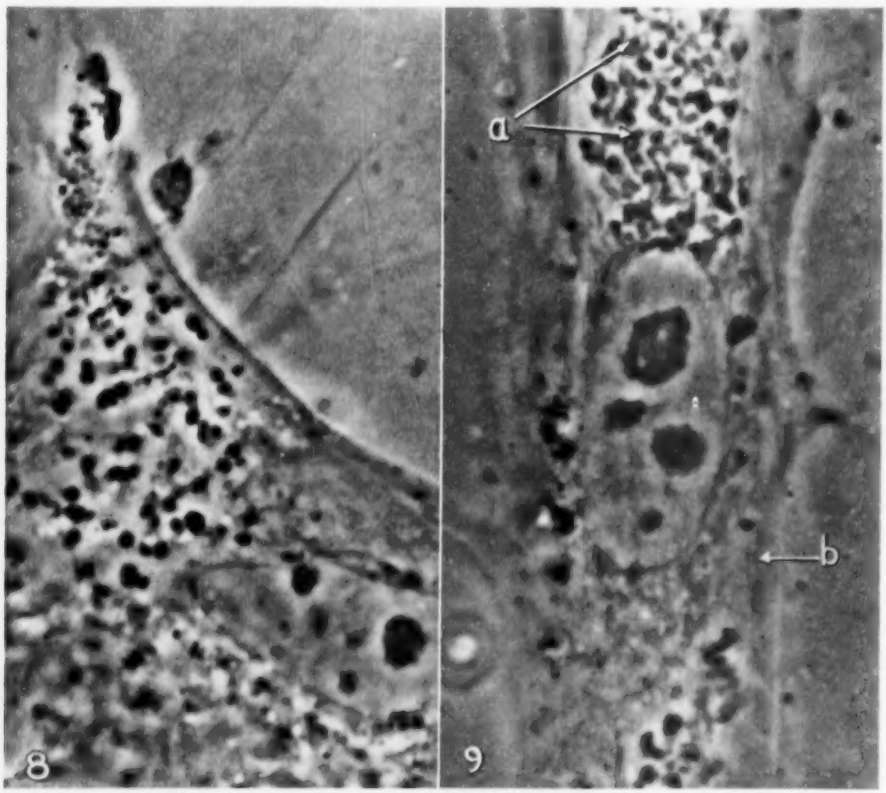


Fig. 8 and 9—Phase-contrast photographs of living cells. Cells of the endothelial spike of figure 7 showing the details of the cells which are in contrast to those of figures 2 and 3. Spheroidal mitochondria (a) and filamentous mitochondrium (b). x 1,718.

showed no cytopathic changes. Equal parts of undiluted serum containing antibodies against HCV were incubated at room temperature (25 C.) for two hours with 10^{-1} or 10^{-2} dilutions of the virus-containing materials. Cells of the outgrowths from cultures exposed to these materials were comparable to the controls.

DISCUSSION

Evidence has been presented to show that tissue cultures from swine embryo lymph nodes exposed to hog cholera virus (HCV) "modified by rabbit passage" (RM), "modified *in vitro*" (MV), or virulent virus (VV) in whole blood or in amnionic fluid showed cytopathic changes of the cytoplasm. These changes were not observed in cultures of cerebellum, kidney, testes, duodenum, spleen, heart, liver, or lung of swine embryo origin. Cultures of choroid plexus showed a noticeable reaction in the second and fourth trials to the presence of virus, which was inconsistent, being simply an erratic pattern of outgrowth among the cultures, most of which were retarded and occasionally showed more granular cytoplasm in comparison to the controls. Lymphocytes died approximately 12 hours following exposure to VV virus in lymph node cultures. Suggestive evidence of this was also developed for similar cultures exposed to all strains of virus at the time of culture.

The first trial demonstrated that there were no highly susceptible cells in the outgrowths from eight source tissues among the ten eventually exposed to virus in these studies. Although it was expected that such might be the case from our earlier work, it was considered that there might well be an optimal time for the uptake or absorption of virus during the development of the outgrowth. It is likely that during pinocytosis virus would be taken into the cells from the medium. If virus were made available during the period of maximum activity of a developing culture, then a maximum exposure of the cells and reactivity could be accomplished. It is possible that cells deep in the clot may escape infection during an optimal period, as considered by Gey and Bang[2]; however, areas of clot liquefaction should expose adequate areas of cells to direct contact with the medium. The second and third trials were unsuccessful in demonstrating an optimal time of exposure in spleen, choroid plexus, or duodenum cultures.

Delayed exposure had been unsuccessful in efforts to demonstrate a cytopathogenic effect of HCV on either cells of fibroblastic reticulae or epithelial sheets. The fourth trial provided exposure of the explants from the time they were cut and put into watch glasses; the fifth trial was designed to confirm the evidence of cytopathogenicity obtained. The effects observed were largely comparable with those found in cultures from an infected animal.[1]

The visible cellular changes were confined to the cytoplasm. The mitochondria became spheroidal; the cytoplasmic volume was reduced, resulting in visible changes in the geography of the cell membranes; and there was increased granularity. These results were considered to be of an order which could become useful for the recognition of the presence of HCV *in vitro* when used with neutralizing antibodies. The observation of death of lymphocytes in perfused cultures of lymph nodes was made possible through time-lapse cinematographic arrangements which also provided a vivid record of the impact of the virus on the cells of the cultures. This observation was not made through the other means of observation utilized, except that "rounded-up" cells were observed to be more numerous in lymph node cultures exposed to all strains of virus (table 4).

The exposed tissue cultures provided wandering cells, fibroblastic reticulae and the attendant endothelial transformations, and epithelial sheets of cells for virus invasion. The changes were found only in the fibroblastic cells and lymphocytes. Epithelial cells from cultures of kidney, ependyma of the choroid plexus, liver, duodenum, and lung were all uniformly refractive to the presence of virus under the conditions of the experiments.

SUMMARY

In the search for a useful cytopathogenic effect of hog cholera virus (HCV), studies were conducted utilizing about 4,400 primary explant tissue cultures representing ten tissues from swine embryos.

The following observations were made:

1) Lymph node cultures exposed prior to being embedded in plasma clots developed cytoplasmic changes in cells of the outgrowth. The mitochondria of the jux-

tanuclear zone were changed to spheroidal shape with clear, light halos; cytoplasmic volume was reduced with attendant bubbling of the cellular membranes and subsequent filamentous strands of attachment to the glass at previous sites of attachment; and there was increased granularity of the cytoplasm. Lymphocytes were observed to die following exposure to virulent HCV.

2) Choroid plexus cultures were retarded in outgrowth grossly and showed inconsistent microscopic changes.

3) Cerebellum, kidney, testes, duodenum, spleen, heart, liver, and lung tissue cultures were not affected measurably by the presence of HCV under the conditions of these studies.

References

[1] Gustafson, D. P., and Pomerat, C. M.: Cytopathic Changes in Tissue Cultures Derived from a Pig Infected with Hog Cholera. Am. J. Vet. Res., *17*, (1956): 165–173.

[2] Gey, G. O., and Bang, F. B.: Viruses and Cells. A Study in Tissue Culture Applications. I. Cells Involved—Availability and Susceptibility. New York Acad. Sci. Tr., *14*, (1951): 15–24.

[3] Orr, M. F., Hardy, M. H., and Pomerat, C. M.: W. Jacobson's Method for Staining Whole Tissue Cultures Differentially for Ribo- and Desoxyribonucleo-Proteins. Abstr. in Anat. Rec., *106*, (1950): 309.

A Modified Assay for Vitamin B_{12} in Swine Blood Serum

G. L. FREDERICK, D.V.M., M.Sc.

Ottawa, Canada

EARLY in the vitamin B_{12} investigations with swine, the need for a blood serum-vitamin B_{12} assay was felt. Previously, *Lactobacillus leichmannii* had been used to assay whole blood [1] and serum [2] from various species of animals including swine. However, the organism, *Euglena gracilis* var. *bacillaris*, seemed more promising, since it was reported to be more sensitive [3] and more specific [4] than *L. leichmannii* for vitamin B_{12}. The assay described by Ross,[3] using *E. gracilis* var. *bacillaris*, is easier to perform than the previous method.

Trial assays with the method of Ross [3] were carried out; these revealed a low percentage recovery of vitamin B_{12} added to swine serum. Pitney *et al.*[5] also used the same method for human serum and reported recoveries of 43 to 65 per cent. They tried two slight modifications of the heat treatment of Ross, but found no significant increase in the recovery. Apparently, the method of freeing the vitamin B_{12} from the serum protein required investigation.[5]

This paper describes a method that gives 100 per cent recovery of vitamin B_{12} added to swine serum.

EXPERIMENTAL PROCEDURE

General.—The apparatus, care of stock cultures, and standards were with a few alterations the same as described by Ross.[3]

Robins *et al.*[6] found that 0.8 per cent of both malic and glutamic acids in their basal medium produced better growth at the higher concentrations of vitamin B_{12}. The medium of Ross [3] was modified accordingly.

The serum treatment was altered. The boiling period was prolonged from 15 minutes to four hours, because this improved the determination of serum vitamin B_{12} and also the recovery of crystalline vitamin B_{12} added to the serum (tables 1 and 3). Centrifugation of the heat-treated, serum-basal medium mixtures at 35,000 r.p.m. for 30 minutes was used for the same reasons (table 2). Potassium cyanide (0.01 mg./ml. of serum) was allowed to react overnight with the serum. This concentration of cyanide was the same as that used by Wolff *et al.*[7] for human serum vitamin B_{12} assays; however, these workers used *L. leichmannii* as the assay organism.

The contents of the vitamin B_{12} standard assay tubes of Ross [3] were modified to contain the same amount of cyanide as the serum assay tubes *(vide infra)*.

The inoculum cells were washed to increase the sensitivity and accuracy of the method.[8]

Titrations were performed with a Beckman model K automatic titrator, using 0.02 N NaOH and an end point of pH 9.6, since Ross [3] had shown that the alkali production by *E. gracilis* var. *bacillaris* is proportional to the amount of growth and to the available vitamin B_{12}.

The tests of the individual serum treatment modifications were performed on serums taken from sows fed an experimental corn-soybean oil meal ration. The final method that combined all the modifications was tested on 25 samples of serum from 10 sows fed the above ration and 11 sows fed the Central Experimental Farm swine herd rations.

Specific.—Reasonable care was taken to prevent bacterial or fungous contamination in the initial proceedings, but strict aseptic technique was used following sterilization of the assay tubes. One milliliter of serum was added to a ⅞- by 6-inch pyrex test tube etched at the 10-ml. level. Vitamin B_{12} solution may be added if required (300 $\mu\mu$g. of vitamin B_{12}/ml. of serum was used in this study). The volume was made up to 5 ml. with double, glass-distilled water. One-tenth milliliter of a potassium cyanide solution containing 10 mg./100 ml. and adjusted to pH 5.1 was added. The tube was shaken, stoppered with nonabsorbent cotton, and the mixture was allowed to stand overnight in the refrigerator.

Five milliliters of full-strength basal medium were added and the tube was restoppered and boiled for four hours. The volume was restored to 10 ml. with double-distilled water. Then, the mixture was shaken and decanted into centrifuge

TABLE 1—Effect of Heat on Recovery of Vitamin B_{12} ($\mu\mu$g. of B_{12}/ml. of Serum*)

	Duration of boiling in minutes					
	Serum A				Serum B	
Sample	15	20	30	45	90	120
Serum	70	150	147	230	11	30
Serum + B_{12} (300 $\mu\mu$g./ml.)	200	319	380	429	253	284
Serum + B_{12} (800 $\mu\mu$g./ml.)	458	557	501	793	378	708

* Each value represents a mean of two separate assays; each assay was conducted in quadruplicate.

Contribution 339, of the Chemistry Division, Science Service, Canada Department of Agriculture.

The author gratefully acknowledges the suggestions and encouragement given by G. J. Brisson and B. B. Migicovsky, and the technical assistance of K. Prasada and G. Sample.

TABLE 2—Effect of Centrifugation on Recovery of Vitamin B_{12} from Serum Boiled for Two Hours with the Basal Medium

Sample	Method	$\mu\mu$g. of B_{12}/ml. of serum *			
		Serum A	Serum B	Serum C	Serum D
1) Serum only	Not centrifuged	0	—	0	0
2) Serum only	Centrifuged	61	0	34	44
3) Serum + B_{12} (300 $\mu\mu$g./ml.)	Not centrifuged	147	121	0	0
4) Serum + B_{12} (300 $\mu\mu$g./ml.)	Centrifuged	275	316	49	45

* Each value represents a mean of two separate assays; each assay was conducted in quadruplicate.

tubes and spun at 35,000 r.p.m. for 30 minutes to remove all coagulated protein and other precipitates. Five milliliters of the supernatant fluid were added to 25-ml. volumetric flasks and made up to volume with half-strength (diluted) basal medium. Aliquots of 4 ml. were pipetted into quadruplicate assay tubes and the tubes were plugged with nonabsorbent cotton. The tubes were inoculated with a five- to seven-day-old dense, washed culture of *E. gracilis* var. *bacillaris* and incubated for five and a half to six and a half days and then titrated.

The volume of the supernatant fluid added to the volumetric flask can be varied to produce the required dilution of the serum.

The standards described by Ross[8] should be prepared with the same amount of potassium cyanide per assay tube, *i.e.*, 0.8 μg. per tube.

Results and Discussion

When this method was used, the mean percentage recovery of vitamin B_{12} added to 25 samples of serum was 101.0 per cent; the standard error of the mean was $\pm$ 3.8. The average difference between 48 replicate assays of serum (with and without added vitamin B_{12}) and their respective means was 6.2 per cent with a standard error of $\pm$ 0.95. There was an inverse relationship between the mean assay values and the differences between replicate assays used to compute the means.

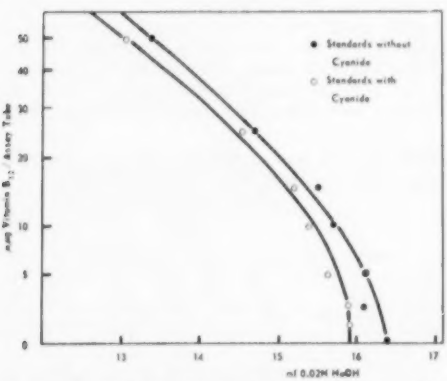


Fig. 1—Comparison of titrations of standards with and without 0.8 μg. of potassium cyanide per tube.

The mean content and the range of serum vitamin B_{12} of the 10 sows fed the corn-soybean oil meal ration were 98 and 18 to 153 $\mu\mu$g./ml., respectively; the values for the serums taken from the 11 sows in the general Central Experimental Farm herd were 167 and 24 to 508 $\mu\mu$g./ml. No definite physiological comparisons can be made between the two groups, because of the uncontrolled dietary regimen of the Farm herd. However, the percentage recoveries of vitamin B_{12} added to the serums were similar in both groups.

TABLE 3—Effect of Cyanide (KCN) and Duration of Boiling (Followed by Centrifugation*) on Determination of Serum Vitamin B_{12} ($\mu\mu$g. of B_{12}/ml. of Serum†)

Animal	Method			
	2 hr. boiling		4 hr. boiling	
	No KCN	KCN	No KCN	KCN
1	51	64	79	111
2	0	8	7	18
3	77	36	162	159
4	34	29	37	38

* Centrifugation at 35,000 r.p.m. for 30 minutes.
† Each value represents a mean of two separate assays; each assay was conducted in quadruplicate.

The data (tables 1 and 3) indicate that heat periods shorter than four hours result in lower determinations of serum vitamin B_{12} and less than 100 per cent recoveries of vitamin B_{12} added to the serum.

Centrifugation improved these two determinations (table 2) by removing the coagulated protein or the other precipitates that were present. The addition of vitamin B_{12} to serums C and D did not produce higher assay values than those obtained for the serums alone. This was probably due to the combining powers of these serums.

Reasons for the use of cyanide were outlined by the Analytical Methods Committee of the Society for Analytical Chemistry.[9] Although the effect of cyanide is not

unequivocal (table 3), its usage in the four-hour boiling method is advocated.

The final concentration of cyanide was considerably greater than any of those that caused growth inhibition in the work reported by Hutner *et al.*[10] The results showed actual stimulation of at least the alkali production in seven out of ten assays. Typical standard curves are shown (fig. 1). The differences in titrations were not due to the alkalinity of the added cyanide. The cyanide did not interfere with the sensitivity of the test. Because the difference in alkali production sometimes significantly influenced the final assay value, one should always use the cyanide-treated standards.

This method did not overcome the "drift," mentioned by Hutner *et al.*,[10] when large variations in serum dilution were used. However, since there was little drift between the dilutions used in this work (0.083 to 0.125 ml. of serum per assay tube) which covered the levels of serum vitamin B_{12} of all the swine studied so far, this may not be important under ordinary conditions.

SUMMARY

A modification is outlined for the blood serum vitamin B_{12} assay of Ross that gives 100 per cent recovery of the vitamin B_{12} added to swine serum and higher serum vitamin B_{12} levels. It utilizes the green alga, *Euglena gracilis* var. *bacillaris*, cyanide treatment of the serum, a four-hour boiling period, and centrifugation.

References

[1] Couch, J. R., Olcese, O., Witten, P. W., and Colby, R. W.: Vitamin B_{12} Content of Blood from Various Species. Am. J. Physiol., *163*, (1950): 77–80.

[2] Wolff, R., and Karlin, R.: Sur la Combinaison du Sérum de Quelques Mammifères avec la Vitamine B_{12}. Bull. Soc. chim. biol., *35*, (1953): 1409–1413.

[3] Ross, G. I. M.: Vitamin B_{12} Assay in Body Fluids Using *Euglena Gracilis*. J. Clin. Path., *5*, (1952): 250–256.

[4] Picken, J. C., Jr., and Bariedel, W. R.: Comparative Bioautography of Vitamin B_{12} and Related Growth Factors Using *Euglena Gracilis* and *Lactobacillus Leichmanii*. Proc. Soc. Exptl. Biol. and Med., *75*, (1950): 511–515.

[5] Pitney, W. R., Beard, M. F., and Van Loon, E. J.: Observations on the Bound Form of Vitamin B_{12} in Human Serum. J. Biol. Chem., *207*, (1954): 143–152.

[6] Robins, W. J., Hervey, A., and Stebbins, M. E.: Euglena and Vitamin B_{12}. An. New York Acad. Sci., *56*, (1953): 818–830.

[7] Wolff, R., Karlin, R., and Paysant, P.: A Propos du Dosage Microbiologique de la Vitamine B_{12} dans le Sérum. Bull. Soc. chim. biol., *37*, (1955): 735–738.

[8] Kristensen, H. P. O.: Investigations into the *Euglena Gracilis* Method for Quantitative Assay of Vitamin B_{12}. Acta Physiol. Scand., *33*, (1955): 232–237.

[9] Analytical Methods Committee of the Society for Analytical Chemistry. Estimation of Vitamin B_{12}. Analyst, *81*, (1956): 132–136.

[10] Hutner, S. H., Bach, M. K., and Ross, G. I. M.: A Sugar-Containing Basal Medium for Vitamin B_{12} Assay with Euglena: Application to Body Fluids. J. Protozool., *3*, (1956): 101–112.

Blood Changes in Swine with African Swine Fever

D. E. DeTRAY, D.V.M., and G. R. SCOTT, M.S., M.R.C.V.S.

Kabete, Kenya, East Africa

AFRICAN SWINE FEVER (ASF) infection in the domestic pig is similar, clinically and pathologically, to hog cholera, although the etiological agents are unrelated immunologically.[1-5] The most pronounced change in the blood picture of swine infected with hog cholera is a leukopenia.[6-14] Likewise, De Kock *et al.*[2] postulated from preliminary observations that leukopenia was a frequent occurrence in ASF, but their published figures do not sufficiently support their hypothesis. The following experiments were undertaken to clarify the blood picture in ASF and to compare the changes with those associated with hog cholera.

MATERIALS AND METHODS

Three Large White swine were studied first, and the observations were then replicated in 3 crossbred black and white swine of unknown ancestry but of European type. All the pigs weighed between 110 and 160 lb. Blood was taken from the anterior vena cava[15,16] each day six hours after feeding. Five milliliters of blood were collected in small bottles containing dried, stock anticoagulant. The anticoagulant was prepared by dissolving 1.2 Gm. of ammonium oxalate and 0.8 Gm. of potassium oxalate in 100 ml. of distilled water. Each collecting bottle held 0.5 ml. of the stock anticoagulant solution, which was then dried slowly at 34 C. Determinations were made on the afternoon of the day of collection and were as follows:

1) Erythrocytes per cubic millimeter (r.b.c.)—0.02 ml. of blood was added to 3.98 ml. of Hayem's solution to make a 1:200 dilution, which was transferred by means of capillary pipette to the improved Neubauer counting chamber.

2) Hemoglobin expressed as grams per 100 ml. of blood—the acid-hematin method was used, the reading being made photoelectrically.

3) Packed cell volume was expressed as millimeters in Wintrobe hematocrit tubes, centrifuged at 3,000 r.p.m. (diameter, 28.8 cm.) for 30 minutes.

4) Leukocytes per cubic millimeter (w.b.c.)—0.1 ml. of blood was added to 1.9 ml. of white cell-diluting fluid to make a 1:20 dilution. The diluting fluid contained 3 per cent glacial acetic acid in distilled water.

5) Differential leukocyte counts—blood smears were treated with Wright's stain. Segmented neutrophils (polymorphonuclears) were classified as mature forms, and all others were regarded as juveniles.

6) Sedimentation rate was expressed as millimeters after one hour in Wintrobe hematocrit tubes.

Determinations were made on each pig for five or six days before inoculation and, thereafter, the observations were continued daily until death. A total of 829 individual observations was analyzed. The virus used was the Hinde, 1954, strain of ASF virus.[17] Each pig received a subcutaneous inoculation of $10^{4.5}$ L.d.$_{50}$ of virus. The accumulated data were subjected to analysis of variance to detect differences between pigs and differences between days. The pre- and postinoculation data were compared by Student's t test. Regressions were tested for curvilinearity and, where appropriate, the polynominal was fitted.

RESULTS

Preinoculation Blood Picture.—The mean preinoculation values, together with the 99 per cent fiducial limits and the range, are recorded (table 1). Significant differences between pigs occurred in the total leukocyte counts ($F = 22.9^{**}$). On the basis of the leukocyte counts, the swine were divisible into two distinct groups. The first group contained the 3 Large White pigs, and the second group consisted of the 3 black and white pigs of unknown breeding. On the basis of the percentage of basophils, 2 pigs had a significantly lower percentage than the rest. Pigs 4, 5, and 6 showed high sedimentation rates (19.9 ± 7.8 mm.). Our other values fall within the accepted limits for normal swine as recorded in the literature (table 2).

Postinoculation Picture.—The mean postinoculation (PI) values are recorded (table 3). Significant differences between pigs occurred in the erythrocyte counts ($F = 6.13^{**}$), which were reflected in the he-

Dr. DeTray is with the Animal Disease and Parasite Research Branch, Agricultural Research Service, U. S. Department of Agriculture, and Mr. Scott is with the Department of Veterinary Services, Kenya, East Africa.

This paper is published under the terms of a memorandum of understanding between the Department of Veterinary Services, Kenya, East Africa, and the Animal Disease and Parasite Research Branch, ARS, U.S.D.A. Permission of both parties to publish is gratefully acknowledged by the authors.

The writers are indebted to Jean DeTray and Betty Rogoff for technical assistance.

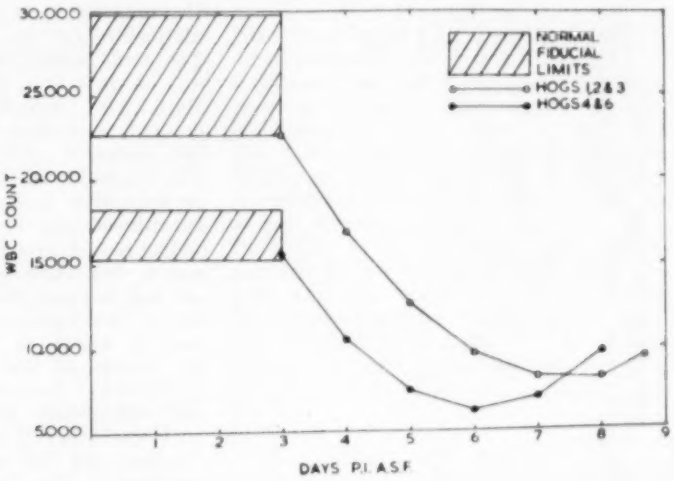


Fig. 1—The fall in the leukocyte counts following exposure to African swine fever.

moglobin values ($F = 4.98$ **) and the packed cell volumes ($F = 4.17$ **). Pig 2 was found to possess a significantly greater number of erythrocytes than the rest. Differences between the rest were negligible. Nucleated erythrocytes, previously absent, appeared in the stained smears beginning on the fourth day after inoculation.

A significant difference between pigs also occurred in the percentage of monocytes ($F = 5.94$ **). Pig 6 had a significantly lower percentage than the others. Significant differences between days after inoculation were detected in the total leukocyte counts ($F = 8.83$ ** and $F = 10.02$ **), the percentage of neutrophils ($F = 2.88$ **), the percentage of juvenile neutrophils ($F = 36.59$ **), and the percentage of lymphocytes ($F = 3.34$ **). On the third day PI, pigs 4, 5, and 6 again showed high sedimentation rates. All other values remained constant and, when compared with preinoculation figures, were found to be similar (table 4).

Total Leukocyte Count.—The preinoculation counts divided the pigs into two groups, which were considered separately. The first group consisted of pigs 1, 2, and 3. After inoculation, there were no significant differences between the 3 pigs. Highly significant differences were apparent, however, between days after inocula-

TABLE 1—The Preinoculation Blood Picture

Method of measurement	No. of observations	Mean		99% fiducial limits	Range	F test of significance of difference between pigs
Red blood count	29	6,000,000	±	450,000	4,390,000 to 7,810,000	1.66
Hemoglobin	25	15.2 Gm.	±	0.4 Gm.	14.0 to 16.4 Gm.	2.01
Packed cell volume	28	44.6 mm.	±	1.0 mm.	41.0 to 50.0 mm.	2.07
Total leukocyte count	[1] 30	21,400	±	2,900	10,600 to 34,900	22.9**
	[2] 15	26,000	±	3,300	19,500 to 34,900	6.23
	[3] 15	16,800	±	1,500	10,600 to 18,400	2.87
Neutrophils	28	32.1%	±	4.5%	17.0 to 52.0%	1.74
Juvenile neutrophils	18	1.2%	±	0.5%	0 to 3.0%	.....
Eosinophils	28	3.4%	±	0.7%	1.0 to 5.5%	1.99
Basophils	[1] 28	0.8%	±	0.4%	0 to 2.5%	4.24**
	[4] 10	0.3%	±	0.4%	0 to 1.0%	0.18
	[5] 18	1.2%	±	0.5%	0 to 2.5%	1.32
Lymphocytes	28	60.6%	±	4.2%	45.0 to 74.0%	2.27
Monocytes	28	3.1%	±	0.5%	1.5 to 5.5%	1.32
Sedimentation rate	[1] 33	12.4 mm.	±	8.1 mm.	0 to 47.0 mm.	7.91**
	[2] 18	2.9 mm.	±	2.3 mm.	0 to 20.0 mm.	1.15
	[3] 15	19.9 mm.	±	7.8 mm.	3.0 to 47.0 mm.	0.71

** Highly significant difference.
[1] All pigs. [2] Pigs 1, 2, and 3. [3] Pigs 4, 5, and 6. [4] Pigs 1 and 2. [5] Pigs 3, 4, 5, and 6.

TABLE 2—Blood Picture Recorded for Normal Swine

Author	Erythrocyte (10^6/cmm.)	Hemoglobin (Gm./100 ml.)	Packed cell volume	Total leukocyte (10^3/cmm.)	Sedimentation rate	Differential leukocyte count in percentages				
						Neutrophils	Eosinophils	Basophils	Lymphocytes	Monocytes
Kernkamp [12]	5.91 (4.17 to 7.8)	9.9 (6.8 to 13.0)	.....	14.2 (8.66 to 23.65)	4.0 (0.1 to 11.0)	37.2	2.8	0.5	54.6	4.0
Lewis and Shope [9]	6.86 (6.1 to 7.22)	.....	.....	21.35 (14.04 to 39.94)	.....	30.5	8.5	1.5	55.5	4.0
Scarborough [28]	6.74	.....	.....	8.2	.....	39.0	4.5	1.2	52.1	3.3
Dukes [21]	7.44	.....	.....	17.11	.....	41.0	2.5	1.0	47.0	8.0
Sikes *et al.* [26]	.....	.....	.....	13.2 to 15.4	<10.0	28.6 to 31.0	0.75 to 3.5	0 to 0.5	62.8 to 70.0	4.5 to 9.3
Albritton [22]	6.93 ♀	13.3 (10.0 to 16.5)	41.5 (30.0 to 53.0)	7.0 to 20.0	5.35 (3.0 to 8.0)	.....	.....	.....	.....	.....
Wintrobe [33]	7.93	15.0	46.3	7.0 to 20.0	.....	.....	.....	.....	.....	.....
Götze [29]	7.09 ♂	13.0	43.2	.....	.....	.....	.....	.....	.....	.....
Götze [29]	6.9 ♀	11.8	38.9	.....	.....	.....	.....	.....	.....	.....
Cahill [5]	.....	.....	.....	13.0 (10.3 to 16.0)	.....	.....	.....	.....	.....	.....
Cole [31]	.....	.....	.....	20.0 (12.0 to 39.0)	.....	.....	.....	.....	.....	.....
King and Wilson [8]	.....	.....	.....	19.98 (10.0 to 39.3)	.....	.....	.....	.....	.....	.....
Sippel [34]	.....	.....	.....	17.5 (14.0 to 40.0)	.....	.....	.....	.....	.....	.....

tion and between the pre- and postinoculation means ($t = 4.776$ **, $P < 0.001$). The total leukocyte count remained normal on days 0, 1, 2, and 3 PI ($t = 1.609$, $P < 0.2 > 0.1$) but, from the fourth day PI onward, a marked and highly significant fall occurred ($t = 10.523$ **, $P < 0.001$). The regression of the total leukocyte count on days after inoculation was found to be curvilinear (fig. 1).

The second group consisted of pigs 4, 5, and 6. Pig 5 showed a marked but transient fall in leukocytes to 5,900 on the third day PI, but otherwise the w.b.c. count remained within the normal limits during the course of the disease ($t = 0.522$, $P < 0.7 > 0.6$). Nevertheless, the changes in the differential counts were consistent with the other 5 swine. Significant differences did not occur between the remaining 2 pigs but, as with the first group, highly significant differences were apparent between counts taken on consecutive days after inoculation and between the pre- and postinoculation means ($t = 3.811$ **, $P < 0.001$). Like the first group, the total leukocyte counts of pigs 4 and 6 remained within the normal limits on days 0, 1, 2, and 3 PI ($t = 0.783$, $P < 0.5 > 0.4$) and fell significantly from the fourth day PI onward ($t = 11.631$ **, $P < 0.001$). Again the line of best fit for the regression of the total leukocyte count on days PI was curvilinear (fig. 1).

The similarity in the response of the leukocytes of the two groups of pigs was emphasized by expressing the fall in the number of leukocytes as a percentage of the normal or preinoculation count (fig. 2). In both groups, the fall was approximately 60 per cent. The curves in figure 2 are statistically identical ($t = 0.599$, $P < 0.6 > 0.5$). The relationship between the leukocyte response and the thermal reaction of the inoculated pigs is depicted (fig. 3). Both the count and the temperature remained within normal limits for the first three days of the infection and both showed significant changes on the fourth day.

Percentage of Neutrophils.—Significant differences in the percentage of neutrophils between the pigs did not occur. The percentage remained unchanged until the fourth day after inoculation ($t = 1.495$, $P < 0.2 > 0.1$). Thereafter, a highly significant increase occurred ($t = 6.057$**, $P < 0.001$). The rise in the percentage of

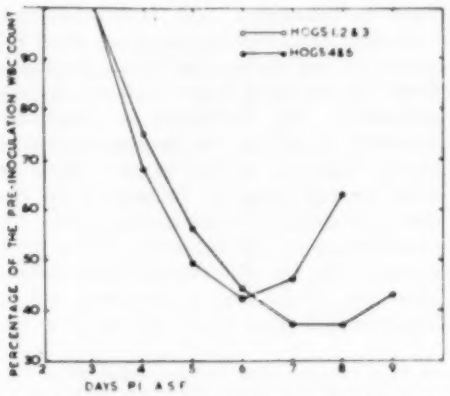


Fig. 2—The percentage fall in leukocyte counts following exposure to African swine fever.

neutrophils was linear on time ($\hat{Y}=27.1+3\cdot3\cdot X$, $r=0.94$), increasing from 37 per cent on the third day PI to 54 per cent on the eighth day PI (fig. 4).

Juvenile Neutrophils.—Correlated with the relative increase in neutrophils was a spectacular increase in the percentage of juvenile forms ($t=6.598^{**}$, $P<0.001$). The percentages of juvenile neutrophils in the normal pigs and for the first three days after inoculation were identical at 1.2 per cent ($t=0.09$, $P>0.9$). Thereafter, the proportion increased linearly ($\hat{Y}=7\cdot3\cdot X-19.6$, $r=0.97$), reaching a maximum

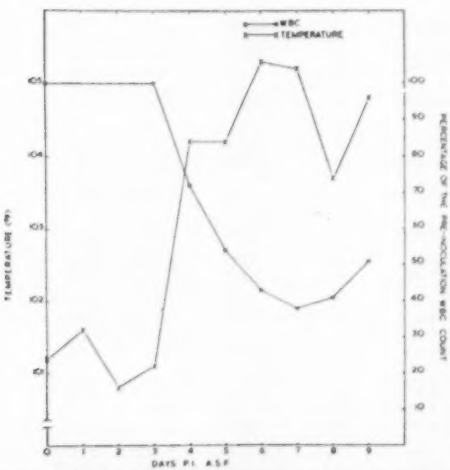


Fig. 3—Relationship between the fall in leukocyte counts and the rise in rectal temperature in African swine fever.

value of 46.0 per cent on the ninth day after inoculation (fig. 5). All the pigs behaved similarly ($F=0.28$).

Percentage of Lymphocytes.—There were no significant differences between the percentage of lymphocytes of the 6 pigs. The percentage increase in neutrophils was mirrored almost exactly by a percentage decrease in the lymphocytes which was linear on days after inoculation ($\hat{Y}=65.7-3\cdot4\cdot X$, $r=0.93$). The percentage fell from a normal of 55.5 to 38.5 per cent (fig. 4). As before, the percentage remained normal for the first three days of the infection ($t=1.826$, $P<0.1>0.05$)

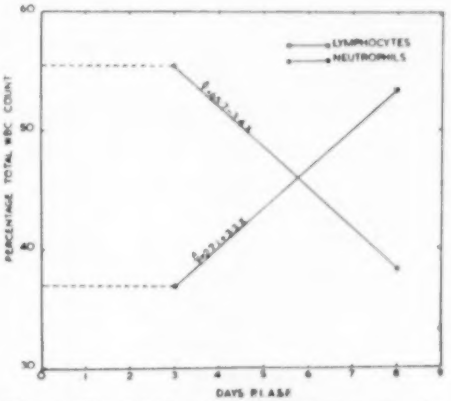


Fig. 4—The highly significant changes in the percentages of lymphocytes and neutrophils following inoculation with African swine fever virus.

but, thereafter, the fall was highly significant ($t=7.004^{**}$, $P<0.001$).

African Swine Fever and Hog Cholera Pictures Compared.—A review of the literature revealed several sets of total leukocyte counts in swine following exposure to hog cholera virus.[9, 11–13] When the data were pooled and analyzed by the same techniques as our ASF figures, highly significant differences occurred between days after exposure ($F=12.4^{**}$). The number remained within normal limits on days 0 and 1 after exposure ($t=0.48$, $P<0.7>0.6$). Thereafter, the highly significant fall began ($F=4.06^{**}$) and the regression of the leukocyte count on days after exposure was curvilinear (fig. 6). The fall occurred two days earlier in hog cholera infections but, thereafter, the values merge and are similar ($t=0.887$, $P<0.4>0.3$). Shu [10]

reported a shift to the left in the neutrophils in hog cholera and Kernkamp [12] reported a reversal in the relationship of neutrophils to lymphocytes as hog cholera infection progressed. Lewis and Shope [9] mentioned that nucleated erythrocytes increased during the course of hog cholera infection. All these changes occurred during ASF infection.

Discussion

The main alterations in the blood picture of pigs infected with ASF were confined to leukocytes. The leukopenia was virtually indistinguishable from that reported in hog cholera infections despite a delay of two days before leukopenia was detectable. This difference is minor because the length of the incubation period or the duration of the eclipse phase of viral multiplication is influenced by the dose and strain of virus administered. An examination of the leukocyte status of a suspect will not differentiate hog cholera from ASF. Indeed, as a diagnostic aid, a single examination is of limited value be-

TABLE 3—The Postinoculation Blood Picture of Pigs Infected with African Swine Fever

Method of measurement	Pigs involved	No. of observations	Mean		99% fiducial limits	F test of significance of difference between—	
						Pigs	Days PI
Red blood count	All pigs	49	5,940,000	±	360,000	6.13**	0.88
	Pigs 1, 3, 4, 5, 6	39	5,700,000	±	310,000	1.05	2.26
	Pig 2	10	7,050,000	±	900,000	.....	.....
Hemoglobin	All pigs	48	15.7 Gm.	±	4.4 Gm.	4.98**	1.81
	Pigs 1, 3, 4, 5, 6	38	15.4 Gm.	±	0.3 Gm.	3.6	2.51
	Pig 2	10	16.8 Gm.	±	1.7 Gm.	.....	.....
Packed cell volume	All pigs	49	46.4 mm.	±	1.4 mm.	4.17**	1.76
	Pigs 1, 3, 4, 5, 6	39	45.6 mm.	±	1.3 mm.	3.23	1.86
	Pig 2	10	49.4 mm.	±	4.6 mm.	.....	.....
White blood count	Pigs 1, 2, 3	26	16,200	±	4,000	1.08	8.83**
	Pigs 1, 2, 3 on days PI 0 to 3	11	23,400	±	3,600	.....	0.49
	Pigs 1, 2, 3 on days PI 4 to 9	15	10,900	±	2,700	.....	1.24
	Pigs 4, 5, 6	24	13,100	±	2,900	2.29	2.62
	Pigs 4, 6	17	11,800	±	3,400	0.37	10.02**
	Pigs 4, 6 on days PI 0 to 3	8	15,900	±	4,400	.....	2.46
	Pigs 4, 6 on days PI 4 to 9	9	8,100	±	1,500	.....	11.04
	Pig 5	7	16,100	±	6,400	.....	.....
Neutrophils	All pigs	50	41.7%	±	3.9%	1.31	2.88**
	All pigs on days PI 0 to 3	23	35.5%	±	4.4%	.....	0.66
	All pigs on days PI 4 to 9	27	47.0%	±	5.2%	.....	0.62
Juvenile neutrophils	All pigs	51	13.1%	±	5.8%	0.28	36.59**
	All pigs on days PI 0 to 3	24	1.2%	±	0.6%	.....	2.01
	All pigs on days PI 4 to 9	27	23.7%	±	7.7%	.....	13.3**
Eosinophils	All pigs	51	3.9%	±	0.7%	2.55	1.26
Basophils	Pigs 1, 2	18	0.9%	±	0.7%	2.54	1.09
	Pigs 3, 4, 5, 6	33	1.0%	±	0.4%	1.59	1.09
Lymphocytes	All pigs	51	50.3%	±	3.8%	1.12	3.34**
	All pigs on days PI 0 to 3	24	56.6%	±	4.4%	.....	0.56
	All pigs on days PI 4 to 9	27	44.7%	±	4.7%	.....	0.7
Monocytes	All pigs	51	3.1%	±	0.3%	5.94**	2.11
	Pigs 1, 2, 3, 4, 5	43	3.3%	±	0.3%	2.41	1.05
	Pig 6	8	2.3%	±	0.9%	.....	.....
Sedimentation rate	Pigs 1, 2, 3	27	6.2 mm.	±	3.6 mm.	1.16	1.43
	Pigs 4, 5, 6	24	10.5 mm.	±	6.0 mm.	0.18	19.85**
	Pigs 4, 5, 6 omitting 3rd day PI	21	5.7 mm.	±	2.8 mm.	.....	2.3

PI = postinoculation.
** Highly significant difference.

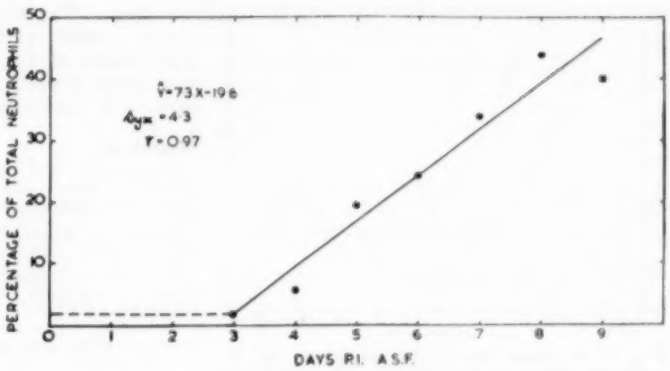


Fig. 5—The highly significant increase in juvenile neutrophils following inoculation with African swine fever virus.

cause of the variations encountered in normal swine blood. A series of observations, preferably daily, are necessary. Admittedly, we would agree with Lewis and Shope [9] that a count of less than 8,000 is suspicious, but a higher count could repre-

TABLE 4—The Comparison of the Pre- and Postinoculation Blood Picture of Pigs Infected with African Swine Fever

Method of measurement	Pigs involved	Preinoculation mean	Postinoculation mean	t	P
Red blood count	Pigs 1, 3, 4, 5, 6	6,000,000	5,700,000	1.749	<0.1 >0.05
	Pig 2	6,800,000	7,050,000	0.717	<0.5 >0.4
Hemoglobin	Pigs 1, 3, 4, 5, 6	15.2 Gm.	15.4 Gm.	0.11	>0.9
	Pig 2	15.7 Gm.	16.8 Gm.	0.146	<0.9 >0.8
Packed cell volume	Pigs 1, 3, 4, 5, 6	44.6 mm.	45.6 mm.	1.58	<0.2 >0.1
	Pig 2	46.3 mm.	49.4 mm.	1.162	<0.3 >0.2
White blood count	Pigs 1, 2, 3	26,000	16,200	4.776**	<0.001
	Pigs 1, 2, 3 on days PI 0 to 3	26,000	23,400	1.609	<0.2 >0.1
	Pigs 1, 2, 3, on days PI 4 to 9	26,000	10,900	10.523**	<0.001
	Pigs 4, 6	16,800	11,800	3.811**	<0.001
	Pigs 4, 6 on days PI 0 to 3	16,800	15,900	0.783	<0.5 >0.4
	Pigs 4, 6 on days PI 4 to 9	16,800	8,100	11.631**	<0.001
	Pig 5	17,200	16,100	0.522	<0.7 >0.6
Neutrophils	All pigs	32.1%	41.7%	4.16**	<0.001
	All pigs on days PI 0 to 3	32.1%	35.5%	1.495	<0.2 >0.1
	All pigs on days PI 4 to 9	32.1%	47.0%	6.057**	<0.001
Juvenile neutrophils	All pigs	1.2%	13.1%	3.262**	<0.01 >0.001
	All pigs on days PI 0 to 3	1.2%	1.2%	0.09	>0.9
	All pigs on days PI 4 to 9	1.2%	23.7%	6.598**	<0.001
Eosinophils	All pigs	3.4%	3.9%	1.211	<0.3 >0.2
Basophils	Pigs 1, 2	0.3%	0.9%	1.966	<0.1 >0.05
	Pigs 3, 4, 5, 6	1.2%	1.0%	0.873	<0.4 >0.3
Lymphocytes	All pigs	60.6%	50.3%	4.617**	<0.001
	All pigs on days PI 0 to 3	60.6%	56.6%	1.826	<0.1 >0.05
	All pigs on days PI 4 to 9	60.6%	44.7%	7.004**	<0.001
Monocytes	Pigs 1, 2, 3, 4, 5	3.1%	3.3%	0.333	<0.8 >0.7
	Pig 6	3.0%	2.3%	1.477	<0.2 >0.1
Sedimentation rate	Pigs 1, 2, 3	2.9 mm.	6.2 mm.	1.446	<0.2 >0.1
	Pigs 4, 5, 6	19.9 mm.	10.5 mm.	1.994	<0.1 >0.05
	Pigs 4, 5, 6 omitting 3rd day PI	19.9 mm.	5.7 mm.	4.438**	<0.001

PI = postinoculation.
** Statistically a highly significant difference.

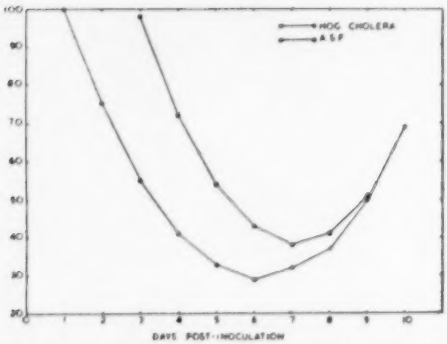


Fig. 6—A comparison of the percentage fall in leukocyte counts in hog cholera and in African swine fever.

sent a relatively greater leukopenia for an individual pig whose normal leukocyte count was high.

As ASF infection progressed beyond the fourth day PI, the lymphocytes were increasingly difficult to identify. Many showed little or no differentiation between nucleus and cytoplasm and often stained as purple blotches. The blotches were believed to be cells that ruptured when the smears were made. The cause was probably cell degeneration leading to increased fragility.

Kernkamp[12] observed a gradual increase in the sedimentation rate during hog cholera infection but Sippel[14] found that it was not sufficiently reliable or definitive to be an aid in the diagnosis of this disease. The pre- and postinoculation sedimentation rates reported here were too variable to be of any value in the diagnosis of ASF. For example, pigs 1, 2, and 3 showed no change, whereas pigs 4, 5, and 6 had transient high sedimentation rates both pre- and postinoculation. The reason for the variations was not apparent and, if they resulted from faulty techniques, the source of the error was not determined.

Summary

Pigs infected with African swine fever (ASF) developed a leukopenia beginning on the fourth day after exposure. The leukopenia was associated with a highly significant increase in the percentage of neutrophils, especially the juvenile forms, and a decrease in the percentage of lymphocytes. The leukopenia was indistinguishable from that associated with hog cholera infections. The erythrocyte counts, hemoglobin values, packed cell volumes, and percentages of eosinophils, basophils, and monocytes remained normal. Sedimentation rates were erratic.

References

[1] Montgomery, R. E.: On a Form of Swine Fever Occurring in British East Africa (Kenya Colony). J. Comp. Path. and Therap., *34*, (1921): 159–191, 243–262.

[2] Walker, J.: East African Swine Fever. Thesis, Univ. Zurich, Baillière, Tindall and Cox, London, 1933.

[3] De Kock, G., Robinson, E. M., and Keppel, J. J. G.: Swine Fever in South Africa. Onderstepoort J. Vet. Sci. and Anim. Indust., *14*, (1940): 31–93.

[4] Conceicao, J. M.: Estudo das Zoonoses Porcinas de Angola, Premiere Relatorio. A Zoonose Porcine Africana de Virus Filtravel (Study of the Diseases of Swine in Angola. I. The African Swine Disease, Due to a Filterable Virus). Angola, Pecuária, Anais dos Serviços de Veterinária e Indústria Animal (1947–1948), *1*, (1949): 217–245.

[5] DeTray, D. E., and Scott, G. R.: The Effect of Hyperimmune Hog Cholera Serum on the Virus of African Swine Fever. J.A.V.M.A., *126*, (1955): 313–314.

[6] King, W. E., and Wilson, R. H.: Studies on Hog Cholera and Preventive Treatment. Kansas Agric. Exper. Sta. Bull. 171, 1910.

[7] Dinwiddie, R. R.: Studies on the Hematology of Normal and Cholera-Infected Hogs. Arkansas Agric. Exper. Sta. Bull. 120, 1914.

[8] Cahill, E. A.: Post-Vaccination Trouble—a Possible Diagnostic Method. J.A.V.M.A., *74*, n.s. *27*, (1929): 425–431.

[9] Lewis, P. A., and Shope, R. E.: The Blood in Hog Cholera. J. Exptl. Med., *50*, (1929): 719–737.

[10] Shu, S.: Studies on the Cellular Changes in Pigs' Blood During the Development of Hog Cholera. Rep. New York State Vet. Coll. (1929–1930): 124–136.

[11] Cole, C. G.: Leucocyte Counts on the Blood of Normal, Cholera-Infected, and Recently Immunized Pigs. J.A.V.M.A., *81*, n.s. *34*, (1932): 392–400.

[12] Kernkamp, H. C. H.: The Blood Picture in Hog Cholera. J.A.V.M.A., *95*, (1939): 525–529.

[13] Dunne, H. W., Smith, E. M., Runnells, R. A., Stafseth, H. J., and Thorp, F., Jr.: A Study of an Encephalitic Strain of Hog Cholera Virus. Am. J. Vet. Res., *13*, (1952): 277–289.

[14] Sippel, W. L.: White Blood Cell Count in Hog Cholera. Vet. Med., *47*, (1952): 497–501.

[15] Carle, B. N., and Dewhirst, W. H., Jr.: A Method for Bleeding Swine. J.A.V.M.A., *101*, (1942): 495–496.

[16] Hoerlein, A. B., Hubbard, E. D., and Getty, R.: The Procurement and Handling of Swine Blood Samples on the Farm. J.A.V.M.A., *119*, (1951): 357–362.

[17] Hammond, R. A., and DeTray, D. E.: A Recent Case of African Swine Fever in Kenya, East Africa. J.A.V.M.A., *126*, (1955): 389–391.

[18] Wintrobe, M. M.: Clinical Hematology. 3rd ed., rev., Lea and Febiger, Philadelphia (1951): 999.

[19] Götze, R.: Quoted by M. M. Wintrobe in "Clinical Hematology." 3rd ed., rev., Lea and Febiger, Philadelphia (1951): 999.

[20] Scarborough, R. A.: Quoted by M. M. Wintrobe in "Clinical Hematology." 3rd ed., rev., Lea and Febiger, Philadelphia (1951): 999.

[21] Dukes, H. H.: The Physiology of Domestic Animals. 4th rev. ed., Comstock Publishing Co., Inc., Ithaca, N. Y., 1937.

[22] Sikes, D., Neher, G. M., and Doyle, L. P.: Studies on Arthritis in Swine. I. Experimental Erysipelas and Chronic Arthritis in Swine. Am. J. Vet. Res., *16*, (1955): 349–366.

[23] Albritton, E. C. (ed.): Standard Values in Blood. W. B. Saunders Co., Philadelphia and London, 1952.

Shackling as a Factor in Ham Hip-Joint Hemorrhage

R. L. KITCHELL, D.V.M., Ph.D.; W. J. AUNAN, Ph.D.; L. W. MURPHY, B.S.; D. P. MOSSBERG, B.S.

St. Paul, Minnesota

MANY UNPUBLISHED SURVEYS conducted by individual packing companies have demonstrated repeatedly that the ham has more costly bruises than any other part of the swine carcass. Data compiled by Livestock Conservation, Inc.,[1] show that 48 per cent of hog bruises occur in the ham. The causes of bruises, as listed by them, is as follows:

Canes, whips, and clubs	42%
Kicking, prodding	20%
Crowding, trampling	15%
Fork and nail punctures	12%
Spreaders	2%
Other causes	9%

No specific studies showing the different types of ham bruises and their causes were available. In visiting various packing plants, the plant personnel constantly referred to a bloody spot on the medial surface of the ham (fig. 8) as the most frequent and most costly bruise.

They referred to this condition as the "flank bruise," "flank strain," "cherry bruise," "internal ham bruise," and by many other names. In all instances, they associated the appearance of the condition with injury to the animal during transit from the farm to the packing plant.

The purpose of this article is to report our findings (1) that this condition is not a conventional bruise but is due to hemorrhage into, and an escape of bloody synovial fluid from, the hip joint into the intermuscular septums of the quadriceps femoris muscle; (2) that the condition is brought about largely by the process of shackling a live hog but also, to a minor extent, by rough handling of the carcass on the gambrels; and (3) that the horizontal sticking and bleeding conveyor method of slaughtering hogs practically eliminates this condition.*

MATERIALS AND METHODS

The studies were conducted by dissecting fresh hams at four plants, identified as plants A, B, C, and D. In plants A and C, the hogs were shackled using the conventional wheel and stirrup method and then exsanguinated while hanging vertically from a rail (fig. 1 and 2). In plant B, the hogs were first immobilized, using a CO_2 unit, then shackled (fig. 3) and exsanguinated while hanging vertically (similar to the method illustrated in fig. 2 except they were immobilized). In plant D, the hogs were immobilized using CO_2, then transferred to a horizontal sticking and bleeding conveyor (fig. 4 to 7). Methods of identifying hams and numbers of hams examined will be presented under results.

RESULTS OF THE INITIAL OBSERVATIONS ON AFFECTED HAMS

Dissection of affected hams revealed that the defect constantly appeared as a bloody spot on the medial surface of the ham, caudal to the sartorius muscle and cranial to the vastus medialis part of the quadriceps femoris muscle (fig. 8). The blood was confined to the intermuscular septums and did not involve the muscle fibers. By dissecting deeper into the ham, a path of blood could be followed in the intermuscular septums to the hip joint. Closer examination revealed that the joint capsule of this joint had been ruptured (fig. 9 and 10). The rupture was usually through a thin portion of the joint capsule which extends distally on the neck of the femur to attach parallel to the origin of the vastus medialis muscle. The more cranial part of the joint capsule, the thickened iliofemoral ligament, was seldom involved. Further ob-

Approved for publication as scientific journal series article 3590, Minnesota Agricultural Experiment Station.

Professor and head, Division of Veterinary Anatomy, School of Veterinary Medicine, University of Minnesota (Kitchell); associate professor, Department of Animal Husbandry, University of Minnesota (Aunan); Service Division, George A. Hormel and Co., Austin, Minn. (Murphy); and regional manager, Northwest Division of Livestock Conservation, Inc., South St. Paul, Minn. (Mossberg).

The authors acknowledge the financial support and cooperation of the following Minnesota packing companies in the initial phases of this work: Armour and Co., South St. Paul; Cudahy Packing Co., Newport; Elliott Packing Co., Duluth; and Swift and Co., South St. Paul, and of George A. Hormel and Co. throughout the entire project.

* A preliminary report of the initial findings was presented at the seventh research conference of the Research Council of the American Meat Institute.[2]

servation showed that the dorsal crus of the round ligament, that part of the ligament which originates from the acetabular fossa, had been torn in part or was entirely free from the periosteum lining the fossa (fig. 11). The ventral crus of the round ligament, which originates largely from the transverse acetabular ligament, had not been torn.

In apparently unblemished hams, blood was present in the synovial fluid in the majority of those examined. In these specimens, the round ligaments had been torn but the joint capsule had not been broken.

These initial observations indicated that the condition was not a bruise but a hemorrhage. Therefore the condition was named "hip-joint hemorrhage" or, technically, "coxofemoral hemarthrosis." Specimens in which blood was present in the synovial fluid but in which the joint capsule had not been broken were classified as "internal hip-joint hemorrhages," while those in which the blood (and synovial fluid) had escaped through the broken joint capsule into the intermuscular septums were classified as "external hip-joint hemorrhages." Three types of the latter were recognized, mild, moderate, and severe (or surface). In the mild type of external hip-joint hemorrhage, the bloody synovial fluid was small in quantity and remained near the tear in the joint capsule. In the moderate type, it was much more extensive in the intermuscular septums but did not reach the surface of the ham. In the severe type, the blood had reached the surface of the ham. The appearance of these hams suggested the various packinghouse terms, such as flank strain, cherry bruise, etc.

Dissection of six latex-injected hams demonstrated that the blood vessels in this region entered through the acetabular notch, deep to the transverse acetabular ligament (fig. 12). The peripheral branches

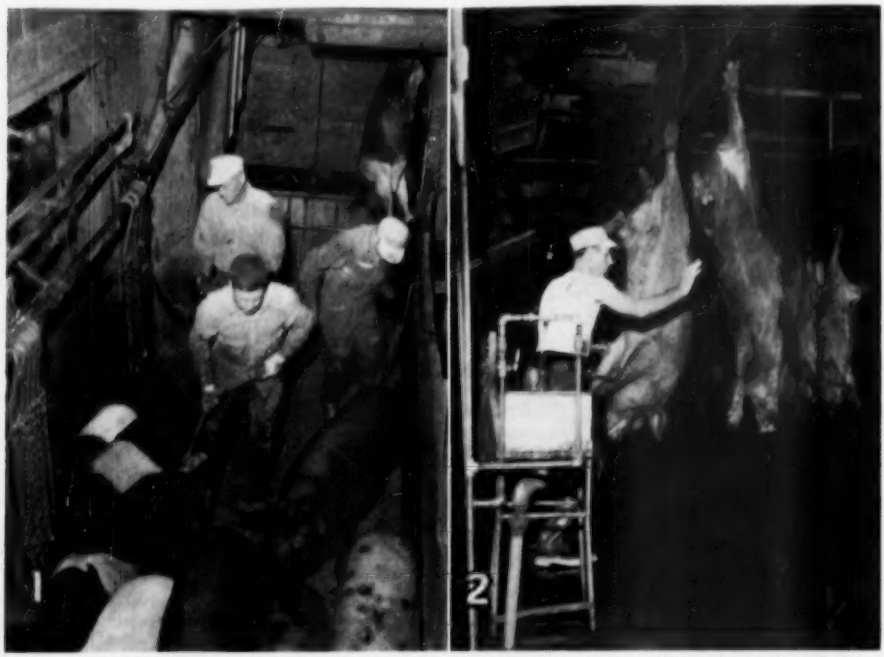


Fig. 1—Conventional wheel and stirrup method of shackling hogs used in plants A and C. The shackling wheel is in the rear hoisting a shackled hog up to a rail (out of the picture).

Fig. 2—Position of hogs during sticking and bleeding in plants A, B, and C. In each plant, the hogs were suspended vertically from a rail by means of a shackle chain attached to one hindleg. In plant B, the hogs were immobilized during sticking as a result of CO_2 inhalation (fig. 3) while, in plant A and C, they were not.

of these vessels ramified in the origin of the dorsal crus of the round ligament. Microscopic studies of the round ligament showed a few blood vessels of significant size which coursed the full length of the ligament.

The above observations led to a consideration of the physical factors which might have been responsible for tearing the round ligament and the joint capsule. In the dissected specimens, when the distal end of the femur was pulled caudally, the caudal edge of the rim of the acetabulum acted as a fulcrum on the neck of the femur. This forced the head of the femur ventromedially, thus tearing the dorsal crus of the round ligament and forcing the head of the femur through the thin part of the capsule. To assist in this phase of the study, radiographs were made of the hindquarters of a hog in a standing position and of a carcass which had been chilled and thus fixed in a shackled position† (fig. 13).

In order to measure the angle of the coxofemoral joint as accurately as possible, a line was drawn on the radiograph through the os coxae, using as landmarks the tuber coxae and the tube ischii, and a second line through the long axis of the femur. The cranioventral angle of intersection of these lines was then measured by means of a protractor. This angle varied between 75 degrees and 90 degrees in the normal standing positions and was 130 degrees in the shackled carcass. The placing of the femur at an angle of 130 degrees in dissected specimens placed considerable tension on the round ligament and the joint capsule. In some instances these structures were torn during this manipulation.

Initial Studies of Defective Hams in Plants A and B

The objectives of these studies were to ascertain the relation of the hemorrhage to the hip joint and its associated structures and to determine whether or not the hemorrhage was related to the shackling procedures.

The first study was conducted in plant A which used the conventional or wheel and stirrup method in their hog-slaughtering operations (fig. 1). The second study was at plant B which immobilized all hogs with CO_2 before shackling and bleeding (fig. 2). In both plants, all hams were identified as from

† Radiographs were prepared by Dr. F. A. Spurrell, Division of Veterinary Surgery and Radiology, School of Veterinary Medicine, St. Paul, Minn.

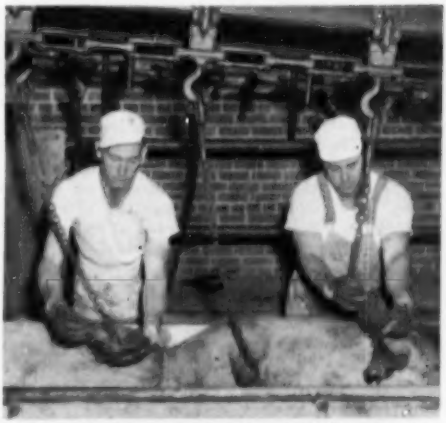


Fig. 3—Shackling method in plant B. The hog has just emerged from the CO_2 immobilizer.

the shackled or nonshackled limb by specific abrasions on the shank produced by the shackling chain. A coded mark was placed on these hams then they followed their normal route to the green grading tables where they were sorted according to weight and quality. All hams showing the severe (surface) type of external hip-joint hemorrhage were placed in separate containers with other defective hams and a few unblemished controls and taken to a boning table.

In an attempt to prevent bias on the part of the

TABLE 1—Data from Plants A and B Showing Incidence of Torn Round Ligaments and Ruptured Joint Capsules as Related to the Type of Defect and to the Shackled or Nonshackled Limb

	No. of hams	Torn ligaments		Ruptured capsules	
		No.	%	No.	%
Plant A—Shackled Limb					
With external hemorrhages	72	66	92	71	99
With other defects	17	12	71	13	76
Without gross defects	3	2	66	1	33
Total	92	80	87	85	92
Plant A—Nonshackled Limb					
With external hemorrhages	10	7	70	10	100
With other defects	9	8	89	5	56
Without gross defects	4	0	0	0	0
Total	23	15	65	15	65
Plant B—Shackled Limb					
With external hemorrhages	355	353	99	342	97
With other defects	34	30	88	11	32
Without gross defects	5	5	100	2	40
Total	394	388	98	355	90
Plant B—Nonshackled Limb					
With external hemorrhages	11	10	91	7	64
With other defects	42	30	71	4	10
Without gross defects	14	7	50	0	0
Total	67	47	70	11	16

research team, one member of the team placed a numbered tag on each ham and recorded the location and extent of any defect on the surface of the ham. He then supervised the removal of the meat and recorded all internal defects. The structures closely related to the hip joint were left intact. The specimen was then examined by the senior author without knowledge as to whether or not a hemorrhage had been present on the surface, or whether it was from the shackled or nonshackled limb. Data recorded at this time included: amount of hemorrhage surrounding the hip joint (external hip-joint hemorrhage), condition of the joint capsules, amount of hemorrhage into the synovial fluid of the joint cavity (internal hip-joint hemorrhage), and condition of the round ligament.

RESULTS OF INITIAL STUDIES

In plant A, 88 per cent of the hams and, in plant B, 97 per cent of the hams with external hip-joint hemorrhages were from the shackled limb (table 2). These data associate shackling with the production of external-hip-joint hemorrhages.

There was an association between torn round ligaments (or ruptured capsules) and external hip-joint hemorrhages (table

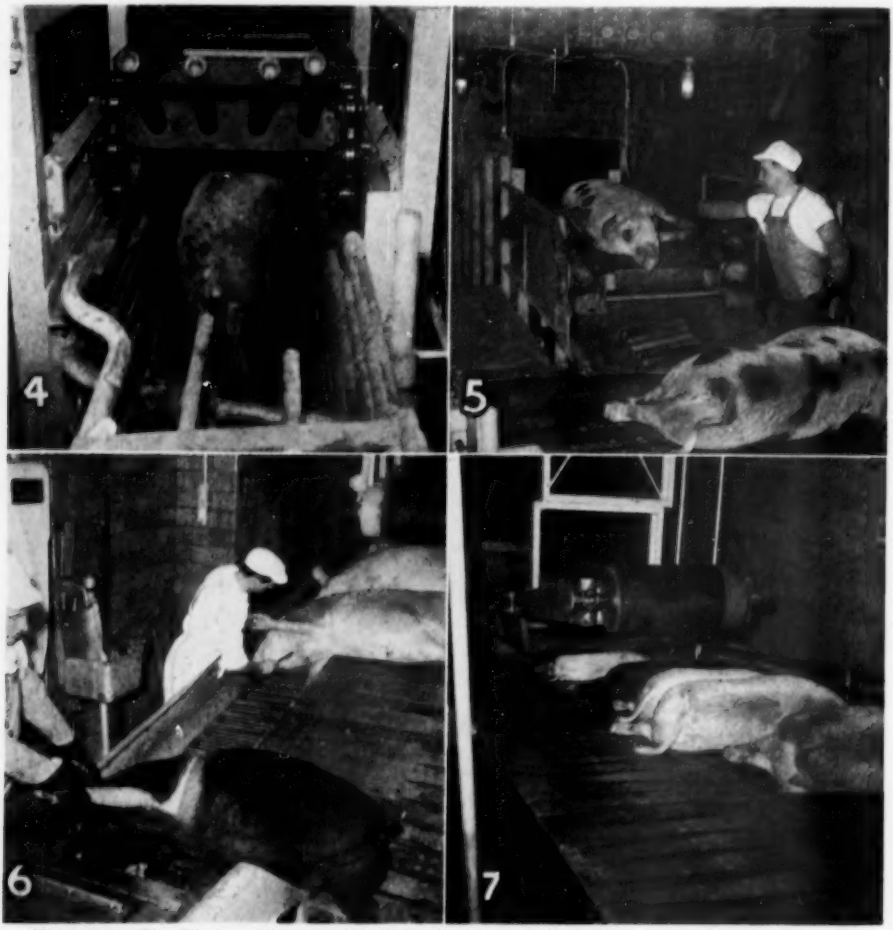


Fig. 4 to 7—The CO_2 immobilizer and horizontal sticking and bleeding conveyor used in plant D. Figure 4 shows the hogs entering the CO_2 immobilizer; figure 5, the immobilized hogs at the exit of the immobilizer; figure 6, the method of positioning and sticking the immobilized hogs; and figure 7, the retainer belt and the killed hogs just before they enter the washing tanks.

TABLE 2—Summary of Data from Table 1 Showing Incidence of External Hip-Joint Hemorrhages in Plants A and B as Related to Shackling

	No. of hams	Shackled limb		Nonshackled limb	
		No.	%	No.	%
Plant A	82	72	88	10	12
Plant B	366	355	97	11	3

3). In plant A, 89 per cent and, in plant B, 99 per cent of the hams with hip-joint hemorrhages also had a torn round ligament. Also, in plant A, 99 per cent and, in plant B, 95 per cent of the hams with external hip-joint hemorrhages had ruptured joint capsules. A much smaller incidence of torn round ligaments and of ruptured capsules can be found in relation to the other defects (tables 1 and 3).

Examination of the totals for hams from the shackled and nonshackled limbs in plants A and B (table 1) shows that an association exists between torn round ligaments and shackling. The incidence of torn round ligaments is considerably higher in the shackled limb (plant A, 87 versus 65%; plant B, 98 versus 70%). Similarly, in both plants the highest incidence of ruptured capsules occurred in hams which were from the shackled leg, thus associating ruptured capsules with shackling.

The observations concerning the amount and character of the fluid in the hip-joint cavity (internal hip-joint hemorrhage) were difficult to make objectively. In instances of external hip-joint hemorrhages, there was little or no fluid remaining in the joint to evaluate. Apparently, movement of the joint during the dressing operations had forced all the residual fluid from the joint. The data from these observations suggest that, in approximately 85 per cent of the unruptured joint capsules, the synovial fluid contained varying amounts of blood, and that there was no appreciable difference between the amount in intact joint capsules of hams from the shackled leg as compared to the nonshackled leg.

TABLE 3—Summary of Data from Table 1 Showing Incidence of Torn Round Ligaments and Ruptured Capsules in Plants A and B as Related to the Type of Defect

	No. of hams	Torn round ligaments		Ruptured capsules	
		No.	%	No.	%
PLANT A					
External hemorrhages	82	73	89	81	99
Other defects	26	20	77	18	69
Without gross defects	7	2	29	1	14
PLANT B					
External hemorrhages	366	363	99	349	95
Other defects	76	60	79	15	20
Without gross defects	19	12	63	2	11

Studies of Hams from Hogs Exsanguinated and then Shackled.—In plant B, 81 CO_2-immobilized hogs were stuck and exsanguinated in the horizontal position, then shackled by one leg, and processed. The hams from this series, along with 82 control hogs which were shackled, exsanquinated, then processed, were identified as to their origin from shackled or nonshackled legs and examined for hip-joint hemorrhages (table 4).

This procedure did not result in a reduction in the external hip-joint hemorrhages in the experimental animals (16%) as compared to their controls (13%). In both groups, the highest number of external hemorrrhages, and all which were

Fig. 8—Medial surface of a ham showing the area where a severe type of external hip-joint hemorrhage appears on the surface of the ham (arrow).

TABLE 4—Comparative Data from Hogs in Plant B Exsanguinated Before Shackling (Experimental Animals) and Hogs Exsanguinated After Shackling (Controls)

	Control series				Experimental series			
	Total No. hams	Percentage of total	Percentage shackled limb	Percentage non-shackled limb	Total No. hams	Percentage of total	Percentage shackled limb	Percentage non-shackled limb
Total hams	164	100	50	50	162	100	50	50
Hams with moderate or severe external hemorrhages	3	2	100	0	3	2	100	0
Hams with mild hemorrhages	19	11	89	11	23	14	73	27
Total hams with external hemorrhages	22	13	91	9	26	16	77	23
Hams with torn ligaments	95	52	65	35	82	50	57	43

severe or moderate, were in the shackled limb. The figures expressed for total external hip-joint hemorrhages also represent ruptured capsules. The incidence of torn round ligaments was significantly greater in the hams from the shackled leg, al-

Fig. 9—The coxofemoral joints of hams with an internal hemorrhage (left) and an external hemorrhage (right). The tear in the joint capsule is distended by a small piece of wood. Note the blood surrounding the joint on the right.

though there were a significant number of torn ligaments in hams from the nonshackled leg.

Studies of Hams from Hogs Exsanguinated and then Shackled by the Front Legs.—In plant B, approximately 50 CO_2-immobilized hogs were exsanguinated, then shackled by the front legs, and processed in the usual manner. This was repeated 12 times. In the hams from these 612 animals, only the presence or absence of the hip-joint hemorrhage was ascertained. In this series of 1,224 hams, only 1.4 per cent had an external hip-joint hemorrhage. Approximately 85.0 per cent had an internal hip-joint hemorrhage.

Studies of Hams from Hogs Exsanguinated and then Shackled by the Front Legs and Never Suspended by the Hind Limbs.—In plant B, 20 CO_2-immobilized hogs were exsanguinated in the horizontal position, shackled after death by the front legs, and processed by being suspended by the front legs. This eliminated the "gambreling" procedure whereby the carcass is suspended by a gambrel inserted in the hindlimbs just prior to cleaning the exterior of the carcass and eviscerating. In none of the 40 hams was the capsule ruptured or any hemorrhage found, either in the intermuscular septums or in the joint cavity itself (fig. 14). One instance of a torn round ligament was observed, but the thickened joint capsule and the character of the tear indicated that it was an antemortem tear which apparently had occurred a considerable time before slaughter.

Studies Conducted in a Plant Using a Sticking and Bleeding Conveyor (Plant D) and a Plant Using Conventional Methods (Plant C).—The following experiment was designed to compare the results of shackling hogs on a hoist without an immobilizer (plant C, fig. 1) and hogs that were immobilized with CO_2 and exsanguinated on a horizontal sticking and bleeding conveyor (plant D, fig. 4 to 7). This conveyor was developed as a result of our initial studies. Under this procedure, shackling was completely omitted but gambreling was not.

One day each month for five consecutive months, about 250 hogs were collected at a buying station and allotted to two comparable groups. One group was shipped to each of two plants. When the hogs were slaughtered, they were marked consecutively by placing a number on each shank. In plant C, an "x" was also marked on the shackled leg and, in plant D, the left leg.

The incidence of external hip-joint hemorrhages (table 5) in plant C was 8.8 per cent and, in plant D, 1.9 per cent. All of the external hip-joint hemorrhages found in hams from plant D were small, whereas many of those in hams from plant C were large. The incidence of internal hip-joint hemorrhages was 85.0 per cent in both plants.

Of the external hip-joint hemorrhages in plant C, 95.0 per cent were in hams from the shackled leg (table 6). The hip-joint hemorrhages in plant D were equally distributed between the right and left legs.

TABLE 6—External Hip-Joint Hemorrhages in Plant C as Related to Shackling

Date	No. of hams	External hemorrhages		
		No.	In shackled leg (%)	In nonshackled leg (%)
December	252	24	88	12
January	244	20	100	0
February	208	26	92	8
March	211	16	100	0
April	235	15	100	0
Total	1,150	101	95	5

DISCUSSION

From these studies, four lines of evidence indicate that the shackling process is primarily responsible for the production of external hip-joint hemorrhages in hams.

TABLE 5—Comparison of Incidence of External Hip-Joint Hemorrhage in Plants C and D

Date	Plant C			Plant D		
	No. of hams	External hemorrhages		No. of hams	External hemorrhages	
		No.	%		No.	%
December	252	24	9.4	254	3	1.2
January	244	20	8.2	237	4	1.7
February	208	26	12.5	210	7	3.3
March	211	16	7.6	208	4	2.0
April	235	15	6.4	231	4	1.7
Total	1,150	101	8.8	1,140	22	1.9

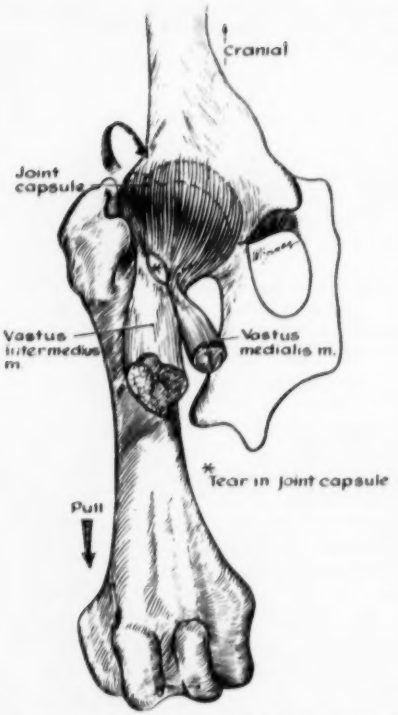


Fig. 10—Drawing of the hip joint of a ham that had an external hemorrhage. Note location of the tear in the joint capsule and its relations to the surrounding muscles.

First is the fact that approximately 95.0 per cent of the external hip-joint hemorrhages were found in hams from the shackled leg (tables 1, 2, 4, and 6). Second, they were fewer in plant D (1.9%) where the shackling process has been eliminated than in plant C (8.8%) which utilizes the

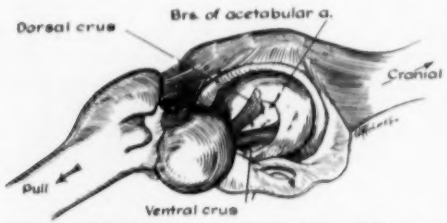


Fig. 11—Drawing of the opened hip joint of a ham that had an external hip-joint hemorrhage. Note the torn dorsal crus, ruptured branches of acetabular vessels, and the tear in the joint capsule (lower arrow).

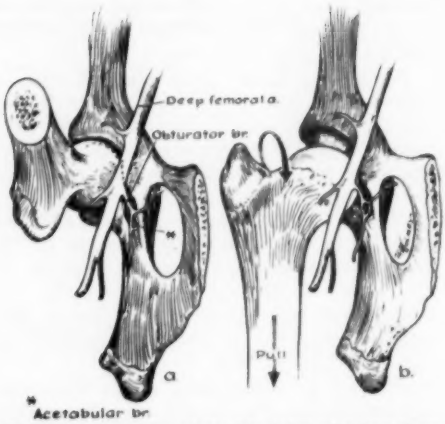


Fig. 12— Drawings of the hip joint of a hog showing the arterial supply to the acetabular fossa and the different relations of the femur in the normal standing position (left) and extended position (right).

shackling procedure (table 5). Third, they were reduced in plant B from 13.0 per cent (table 4) to 1.4 per cent through shackling the hogs by the forelegs after horizontal sticking. Fourth, they were completely absent in hogs which were neither shackled nor gambreled by their hindlegs.

The studies also show that a small number of external hip-joint hemorrhages are produced by factors (or a factor) other than shackling. This is indicated by the

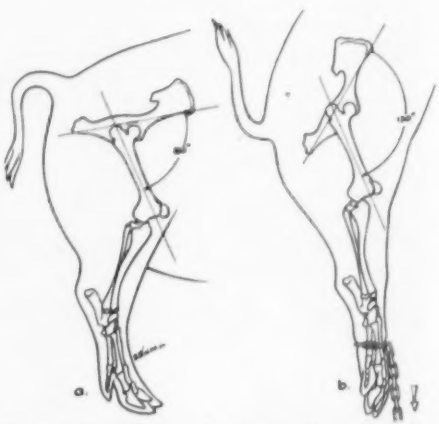


Fig. 13—Drawings made from radiographs of a hog in a standing position (a) and of a split carcass in a shackled position (b). Note increase in the angle of the hip joint in the shackled limb.

observation that in plant D the elimination of shackling did not result in an elimination of all hip-joint hemorrhages—1.9 per cent of all hams still had the mild type. Similarly, in plant B, the experimental procedure of shackling by the front legs did not eliminate all of the hip-joint hemorrhages but reduced them from 13.0 per cent (table 4) to 1.4 per cent.

Also suggestive of additional factors was the presence of some mild types of external hip-joint hemorrhages in hams from the nonshackled limb (plant A, 12.0% of all hams showing external hip-joint hemorrhages; plant B, 9.0%; plant C, 5.0%). Assuming that these factors would affect the ham from the shackled leg as well as from the nonshackled leg, the results would indicate that in plant A, 24.0 per cent; in plant B, 18.0 per cent; and in plant C, 10.0 per cent of all hip-joint hemorrhages in hams were due to factors other than shackling. Using this same assumption and data for plant C where all hams of the series were examined (table 5), it can be calculated that in this plant 0.9 per cent of all hams processed had external hip-joint hemorrhages due to factors other than shackling. This can be compared to 1.4 per cent (shackled by front leg series) for plant B and 1.9 per cent for plant D.

The complete absence of external hip-joint hemorrhages in hams from hogs neither shackled nor gambreled on the hindlimbs is strong evidence that gambreling is the most outstanding of the possible factors, other than shackling, which pro-

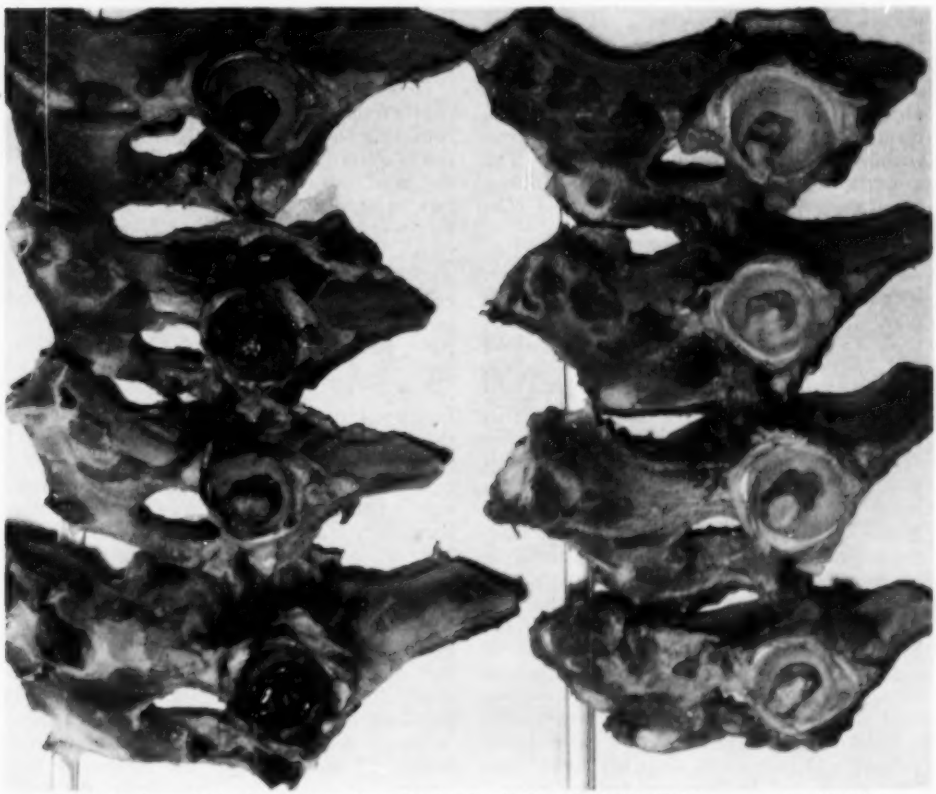


Fig. 14—Opened hip joints from hogs shackled and "gambreled" (left) compared with hip joints from hogs neither shackled nor gambreled (right). Note presence of blood (internal hip-joint hemorrhage) in the synovial fluid of the hip joints from the shackled and gambreled hogs.

duce external hip-joint hemorrhages in hams.

These observations and data suggest how shackling actually produces a hip-joint hemorrhage. More ruptured joint capsules and torn round ligaments are present in the shackled than the nonshackled limb. Also, the hemorrhages from the shackled limb are larger (moderate and severe), whereas those from the nonshackled limb are small (mild). This suggests that the shackling process is significant in the production of external hip-joint hemorrhages, not just because it tears the round ligaments and the joint capsules, but because it tears them while the animals are still alive, resulting in hemorrhage from the torn blood vessels in the origin of the round ligament. If the joint capsule should remain intact, this would result in an internal hip-joint hemorrhage. However, if the shock of shackling is great enough, the joint capsule may be simultaneously or subsequently torn while the animals are still alive. The blood, and the synovial fluid, escape into the intermuscular septums through the tear in the joint capsule. The quantity of blood present, plus continuing hemorrhage, results in a hematoma of considerable size.

That gambreling is largely responsible for internal hip-joint hemorrhage is shown by two lines of evidence. First is the complete absence of internal hip-joint hemorrhages when the hogs are neither shackled nor gambreled. Second is that the elimination of shackling (plant D, and shackling by the forelegs, plant B) did not result in an appreciable reduction in the incidence of internal hip-joint hemorrhages. Suggestive evidence that shackling may produce some internal hip-joint hemorrhage is the larger number of torn round ligaments in the shackled leg as compared to the nonshackled leg (tables 1, 3, and 4).

The above observations suggest that the gambreling process produces an internal hemorrhage as follows: At the time of gambreling, the weight of the carcass and the stress of dropping rapidly on the gambrel result in the tearing of a large number of round ligaments and their blood vessels. Although the animals have been bled out and their heart action has stopped, a quantity of blood remains in their vascular systems. When the carcass is suspended by the gambrel, its weight is transferred to muscles of the hindlimb and the skin is tightened. It would appear that this could apply pressure to the residual blood in the vascular system of the ham and force the blood through the torn vessels into the joint. If the forces applied during gambreling are great enough, a few joint capsules may also be torn permitting the bloody synovial fluid to escape and thus producing an external hip-joint hemorrhage. However, since the heart action has stopped, the amount of blood extruded from the vascular system is relatively small and is not as damaging to the quality of the ham as are those hemorrhages due to shackling.

In determining why certain animals (approximately 18%) develop external hip-joint hemorrhages in response to shackling, whereas other animals (82%) do not, several factors need to be considered. First, a living animal reacts to an externally induced change in the position of a part of its body by muscular activity.

In almost all the joints of the body, and particularly the hip joint, the muscles surrounding the joint actively support it and resist external forces acting on it. When the hindlimb is stretched or moved, receptors located in the muscles, tendons, and ligaments are activated and sensory impulses are directed toward the spinal cord where they are integrated and the result relayed to motor neurons which send impulses to the proper muscles so as to oppose the externally applied force. This reaction by the nerves and muscles is referred to as the "stretch reflex." It functions in such a manner that the greater the external force stretching the muscles, the more stronger the muscles will be stimulated to contract, the ligaments playing only a passive role. Therefore the ligaments surrounding a joint usually are stretched only when the stress of the external force is not fully compensated by muscular action. This may occur if the muscles are inherently weak, the stress is unusually great, the stress continues until the muscles are fatigued, the stress occurs too suddenly for the stretch reflex to protect the joint, or for some reason the stretch reflex is unable to be elicited when stress is applied. If the stress on the ligaments becomes too great, they may tear, as they are inelastic.

In the shackled hog, any sudden jarring might tear the round ligament and joint capsule, as it usually occurs too rapidly

for the stretch reflex to compensate by producing a muscular contraction. Other possible factors could be a turning or struggling of the hog with a sudden relaxation of the muscles in the shackled leg and, also, any incident which would result in the live hog being suspended for a considerable period of time before sticking might increase the possibility of tearing the ligaments, as the muscles would become fatigued. In instances where CO_2 is used to immobilize the hogs prior to shackling, too much CO_2 might depress the nervous system and interfere with the stretch reflex. Other factors such as variations in skeletal structure, disease, and fatigue from handling before shackling could theoretically be involved.

The economic significance of these defects involves several factors. The actual loss to an individual packing company is difficult to ascertain, since many hams showing these defects are boned, thus only the trimmed-away bloody meat is a loss. Of the hams having external hip-joint hemorrhages, less than 20.0 per cent are of the severe type, thus the hemorrhage is not visible on the surface. Also, it is logical to assume that a certain percentage of regular hams (cured, smoked, and sold whole) have external hip-joint hemorrhages of the moderate and mild types. When consumer acceptance is considered, these may prove to be more economically significant than the hemorrhages found prior to selling the product. The percentage of claims for defective hams due to this condition has not been ascertained.

Reports from cooperating plants have shown that the loss involved in processing a ham with external hip-joint hemorrhage is somewhere between $.50 and $2.50 per ham, depending upon wholesale ham values and the method of calculation. Using a conservative estimate of the loss, $1.50 per ham, and a conservative incidence of 2.0 per cent for all hams so affected, the estimated loss for a plant which processes 10,000 hams daily would be $300 per day due to this type of defect. The annual loss for such a plant would be approximately $90,000 a year. On a national basis, the annual loss may be estimated by using the total number of hogs slaughtered under federal inspection during 1954. Based on 52,893,863 hogs slaughtered, this loss would have amounted to $3,173,633. If one estimated a loss of $.50 per ham with any type of external hip-joint hemorrhage, and the incidence determined by this study (8.8%, table 4), the loss to a plant processing 10,000 hams each day would be $440 a day or $132,000 a year. During 1954, the national loss would have amounted to $4,664,000.

It is evident from these studies that the horizontal sticking and bleeding conveyor practically eliminates the loss from this condition. It resulted in an incidence of this defect in plant D of 1.9 per cent as compared to 8.8 per cent in plant C, which uses conventional methods. It eliminated the severe and moderate types of external hemorrhages. In order to further reduce the external hip-joint hemorrhages below 1.9 per cent and the internal hip-joint hemorrhages, attention will have to be directed toward improving or eliminating the gambreling procedure.

Summary and Conclusions

In studies conducted in four widely separated packing plants, evidence has been presented which indicates that shackling is responsible for production of most of the external hip-joint hemorrhages in hams. Additional evidence suggests that the "gambreling" process may be largely responsible for the remainder. The studies also show that the gambreling is responsible for production of most of the internal hip-joint hemorrhages. The horizontal sticking and bleeding conveyor method of slaughtering hogs practically eliminates losses due to external hip-joint hemorrhages.

References

[1] Livestock Conservation, Inc.: Livestock Conservation Handbook. Livestock Conservation, Inc., Chicago (1955): 6–7.

[2] Kitchell, R. L., Aunan, W. J., Mossberg, D. P., and Anderson, P. A.: Studies of Factors Responsible for Internal Hemorrhages in Hams. Proc. Seventh Res. Conf. Am. Meat Inst. (1955): 11–20.

The *in Vitro* Growth of Hog Cholera Virus in Cells of Peripheral Blood

H. W. DUNNE, D.V.M., Ph.D.; A. J. LUEDKE, D.V.M.; C. V. REICH, M.S.; J. F. HOKANSON, D.V.M.

University Park, Pennsylvania

ONE of the major problems in the propagation of viruses *in vitro* is the acquisition of a suitable tissue, under sterile conditions, at the time it is needed and at minimum cost. Slaughterhouses are not always conveniently located, may not be slaughtering the desired species or type at the time, and offer poor conditions under which to obtain sterile material. The destruction of an animal in the laboratory for a single tissue is often expensive.

If the virus of hog cholera could be grown readily in blood cells, there would be available a source of cells which would be dependable, convenient, and relatively inexpensive. The tissue could be obtained under sterile conditions and would not require the killing of the animal. One animal could provide a cell source for a large number of continuing experiments; the cost would be limited to the original purchase price and the expense of feed and handling. This, of course, would be of particular value to the individual research scientist with moderate capital. It also could be of importance in commercial enterprise.

REVIEW OF LITERATURE

For many years, it has been known that hog cholera caused a marked reduction in the number of leukocytes in the circulating blood.[1-5] This leukopenia was shown to be caused by a decrease in both granulocytes and lymphocytes and was accompanied by a shift to young and immature forms.[6]

The adherence of the virus of hog cholera to cells of the blood was shown by several workers[7-9] but hemagglutination was never demonstrated.

The cultivation of fowl plague virus in cells from defibrinated fowl blood by Marchorex and the growth of Newcastle disease virus in defibrinated chicken blood by Dinter and Dinter were cited by Traub.[10]

Traub[10-12] confirmed the work of Dinter and Dinter and showed that the blood cells are not easily infected, the erythrocyte is not the host cell, and that hemolysates have a stabilizing effect on viruses. He further felt that the monocyte was the cell necessary for virus growth.

MATERIALS AND METHODS

The viruses used in these experiments were from two sources. One was a Michigan strain referred to previously by Dunne *et al.*[13] as the "Michigan A" virus. This virus had been stored at −10 C. for 26 months following 46 intracranial passages in swine. It was passed through one swine passage prior to its use in this experiment. Blood virus thus obtained titered approximately 1×10^{-7} in swine. The second virus, which was called the "Sprankle" strain, was isolated in Pennsylvania one month prior to its first use in tissue culture.

The cells used for virus propagation were obtained from the blood of nonimmunized swine, 8 to 16 weeks old. Two or more pigs were maintained in isolation as the source of normal blood. These pigs were from immune sows and were weaned a minimum of ten days prior to bleeding.

All swine blood was obtained by bleeding from the anterior vena cava using a 50- or 100-ml. syringe and a 16-gauge, 3-inch needle. The blood was placed in an 8-oz. jar and defibrinated by agitation. The fibrin was removed by straining through two thicknesses of sterile cheese cloth. To 100 ml. of defibrinated blood, 10 mg. of dihydrostreptomycin and 1,000 units of crystalline sodium penicillin G were added.

Three basic culture systems were used with the Michigan A virus. One system utilized all the cells of the blood. A second system incorporated the leukocytes with a minimum number of erythrocytes. The third system used only the erythrocytes. Only the first two basic systems were used with the Sprankle virus in the second phase of this experiment.

In cultures using all the blood cells, defibrinated whole blood comprised the cell suspension. In the second basic system, where the leukocytes were utilized with a minimum number of erythrocytes, separation was accomplished by centrifugation and aspiration of the serum and then of the leukocyte layer. A second and third centrifugation of the aspirated material removed all but approximately 5 per cent of the erythrocytes. Erythrocytes as a sole source of cells for virus propagation were also obtained by centrifugation and aspiration. In all cases, the serum aspirated, less a small

Authorized for publication on Jan. 5, 1957, as paper 2117 in the journal series of the Pennsylvania Agricultural Experiment Station. From the Department of Veterinary Science, Pennsylvania State University, University Park.

The authors gratefully acknowledge the technical assistance of T. V. Graham and E. H. Ammerman.

amount aspirated with the leukocyte layer, was returned in full volume to the cells to be used in culture. The cell suspensions of all three systems were diluted with an equal portion of Tyrode's basic salt solution.[18]

The diluted cell suspension was placed in water dilution bottles in 10.0-ml. quantities. To this suspension was added 0.1 ml. of the virus. Cultures were incubated at 37 C. for three days without agitation. Upon harvest, the subsequent passage was usually inoculated immediately. When this was not possible, the suspension was placed into 10.0-ml. vials, sealed, and frozen until used in the next passage.

Detection of the virus in cultures was made by swine inoculation. Infection in the animal was determined primarily by a rise in temperature to 104 F. or above and a marked drop in the total leukocyte count. The diagnosis of hog cholera was aided by observations of anorexia, inactivity, unsteady gait, discharge from the eyes, and diarrhea. Temperatures were taken at the time of inoculation and daily thereafter. Leukocyte counts were made at the time of inoculation and in most cases daily from three to ten days postinoculation. All surviving animals were challenged with lethal hog cholera virus.

The pigs used in the virus-detection tests were raised at the University. With few exceptions, all test pigs were from hog cholera-immune sows and were weaned a minimum of ten days before exposure to hog cholera virus.

RESULTS

In the first phase of this research, the serial passage of hog cholera virus in cells from peripheral blood was made using the Michigan A strain of hog cholera virus in cultures of defibrinated whole blood diluted with an equal portion of Tyrode's solution. A positive test for lethal virus was obtained throughout 18 passages of the virus in the cell culture (table 1). Recognizing that each culture represented a virus dilution of 1:100, this would mean a dilution of 1×10^{-36} of the original virus at the eighteenth passage. From the fifth passage, where the inoculum dilution factor reached 1×10^{-10}, it was obvious that virus propagation had been obtained. Cultures were positive for lethal virus in susceptible swine in passages 2, 4, 5, 7, 9, 14, 16, and 18. Titers ranged from 1×10^{-4} through 1×10^{-6}. None of the infected animals recovered from the disease. All inoculated test animals died or were destroyed in a moribund condition. Although the Michigan A virus had previously shown convulsion-producing activity, none of the animals infected with the cultured virus showed severe central nervous system disturbances. The titer of the virus at passage 18, using defibrinated whole blood cultures, was more than 1×10^{-6}.

At passage 7, when it was certain that virus propagation had been obtained in defibrinated whole blood cultures, it seemed desirable to know which cells were responsible for the growth of the virus. Therefore, cultures utilizing primarily the leukocytes and cultures utilizing only the erythrocytes were made to show comparative virus growth. Cultures utilizing the leukocytes were positive for lethal virus through 11 additional passages when inoculated into susceptible swine (table 2). Cultures utilizing erythrocytes alone, however, were negative after five additional serial passages. It was apparent that virus propagation was directly associated with the leukocytes in the cultures.

At passage 26, the Michigan A virus in both the defibrinated whole blood series and the leukocyte series failed to demonstrate lethality in inoculated swine. All swine injected with these culture viruses demon-

TABLE 1—Infectivity of Michigan A Virus Propagated in Defibrinated Whole Blood Cultures

Passage No.	No. of pigs	Reaction in pigs: Highest positive dilution	Fever	Leukopenia	Comments	Deaths	Challenge deaths
2	2	1×10^{0}*	+++	+++	.....	2	.....
4	1	1×10^{0}*	+++	+++	.....	1	.....
5	3	1×10^{-5}	+++	+++	.....	3	.....
7	4	1×10^{-6}	+++	+++	.....	4	.....
9	2	1×10^{-4}	+++	+++	.....	2	.....
14	3	1×10^{-4}	+++	+++	.....	3	.....
16	3	1×10^{-5}	+++	+++	.....	3	.....
18	4	1×10^{-6}	+++	+++	4/4 severe diarrhea	4	.....
22	2	1×10^{0}*	++	++	.....	0	2/2
26	4	1×10^{-6}	++	++	2/4 severe diarrhea	1†	3/3
30	3	1×10^{-2}	+	++	.....	0	3/3

+++ = severe reaction; ++ = moderate reaction; + = mild reaction.
* Only dilution tested. † Died from a suppurative pneumonia.

TABLE 2—Infectivity of Michigan A Virus Propagated in Leukocyte Cultures

Passage No.	No. of pigs	Reaction in pigs: Highest positive dilution	Fever	Leukopenia	Comments	Deaths	Challenge deaths
18	3	1×10^{-6}	+++	+++	.....	3	.....
22	2	$1 \times 10^{0*}$	++	+++	.....	0	2/2
26	2	1×10^{-3}	++	++	.....	0	2/2
30	5	1×10^{-6}	+	+++	4/5 severe diarrhea	1	4/4
43	4	$1 \times 10^{0*}$	+	+	.....	0	3/3

+++ = severe reaction; ++ = moderate reaction; + = mild reaction.

* Only dilution tested.

strated a typical leukopenia on the third to fourth day. A mild temperature rise and in many cases a marked diarrhea appeared on the succeeding three to four days, followed by complete recovery. However, subsequent challenge of these recovered animals, with 1.0 ml. of undiluted virulent blood virus, subcutaneously, resulted in 100 per cent mortality. At passage 30 in the defibrinated whole blood series, the only reactions observed in inoculated animals were a mild temperature reaction and a slight drop in the total leukocyte count. In the culture series utilizing primarily the leukocytes, the reaction, at passage 30, was characterized by a marked leukopenia and severe diarrhea. None of this culture material produced a protective immunity in hog cholera-susceptible swine. None of the animals inoculated with material from either of the two Michigan A virus series were immune when challenged with virulent virus. However, proof of subprotective immunity was established when swine, which were inoculated with Michigan A virus propagated in leukocytes, were protected against virulent hog cholera virus in a delayed serum test.

The delayed serum test for subprotective immunity (table 3) was based on the fact that hog cholera infection can be treated successfully with anti-hog cholera serum only when serum is given within a specific period following infection and in sufficient amounts. Experimentally, this period is usually no longer than three days. The test herein described was conducted for the determination of subprotective immunity produced by the nonlethal virus grown in the leukocyte cultures. It is possible that animals injected with this virus culture may have been partially immunized but, when subsequently challenged with lethal virus, protection was insufficient to prevent hog cholera from progressing through an acute course. Treatment of similar animals with anti-hog cholera serum after the third day following challenge would not be effective unless additional protection was provided from some other source, *i.e.*, active immunity. Three pigs which had received nonlethal Michigan A virus from passages 25, 27, and 29, more than three weeks prior to the test, were challenged with lethal hog cholera virus. Three control pigs which had received no previous

TABLE 3—Subprotective Immunity Produced by an Altered Virus Demonstrated by Delayed Serum Injections

Pig No.	Initial virus exposure	Challenge dose of virulent blood virus	Reaction to challenge	Serum given (days after challenge)	Reaction
533	25 and 27 passage	1.0 ml. undiluted	+++	4	Complete recovery
524	29 passage	1.0 ml. undiluted	+++	5	Chronic, recovered
529	25 and 27 passage	1.0 ml. undiluted	+++	6	Complete recovery
		CONTROLS			
549	None	1.0 ml. diluted, 1×10^{-3}	+++	4	Chronic, stunted
551	None	1.0 ml. diluted, 1×10^{-3}	+++	5	Died in 10 days
553	None	1.0 ml. diluted, 1×10^{-3}	+++	6	Died in 13 days

+++ = severe reaction.

Serum dose: 1.5 ml. per pound of body weight.

TABLE 4—Infectivity of Sprankle Virus Propagated in Defibrinated Whole Blood Cultures

Passage No.	No. of pigs	Reaction in pigs				Challenge deaths
		Highest positive dilution	Fever	Leukopenia	Deaths	
8	1	Undiluted	+++	+++	0	Immune
23	2	Undiluted	+	+	0	2/2

+++ = severe reaction; + = mild reaction.

exposure to a virus of any type also were challenged with lethal virus.

On the fourth day following challenge and after each challenged animal had shown an increase in temperature to 104 F. or higher, anti-hog cholera serum was given to 1 animal from each of the two groups. Serum was given on the fifth day to another pig from each group and on the sixth day to the remaining 2 pigs, the dosage being approximately 1.5 ml. per pound of body weight (table 3). All 3 pigs which previously had been exposed to the nonlethal, nonprotective, tissue culture virus were successfully treated when given serum as late as six days after challenge. Although one developed a somewhat chronic disease, it did not become a runt. Of the 3 control pigs which had no previous exposure to a nonlethal virus, only the 1 which received the virus on the fourth day survived. It remained a runt until destroyed.

Thus, it appeared that between passages 18 and 22 there was an alteration of both pathogenicity and antigenicity. The immunity developed with this virus was not sufficient to protect against death from lethal virus without the aid of additional protective serum.

The second phase of this experiment was designed to show the reaction of a virus strain from another source (Sprankle virus).

Two series of passages were made, using Sprankle virus in the same systems used for the cultures previously described. As in the first phase of this report, one culture system utilized both erythrocytes and leukocytes, whereas the second system used leukocytes with 95 per cent of the erythrocytes removed. Both series were checked for infectivity at the eighth passage, using 1 pig to check each series. Normal leukocyte counts on these animals were 17,900 and 18,100, respectively. Within five days after inoculation, the susceptible pigs in each series showed temperatures of 106.8 and 107.4 F. and a leukopenia as low as 3,500. Both animals recovered and were immune to subsequent challenge with virulent virus. The two series were tested again at passage 23. The undiluted culture from the series utilizing both erythrocytes and leukocytes produced little or no reaction when 1.0 ml. was inoculated into swine. These animals, however, were susceptible to subsequent challenge with virulent virus. Inoculation with passage 23 virus, cultivated in leukocytes, resulted in a leukopenia of 2,500 in 1 pig and 6,850 in the other. Their previous normal leukocyte counts had been 18,700 and 19,000, respectively. The highest temperature was 107.4 in the first pig and 103.8 F. in the second. Both pigs recovered and were subsequently immune to challenge.

Passage 24 virus was grown in a 500-ml. Roux flask containing a total of 50 ml. of culture with all materials in the same relative proportion as was used in the water

TABLE 5—Infectivity of Sprankle Virus Propagated in Leukocyte Cultures

Passage No.	No. of pigs	Reaction in pigs					Challenge deaths
		Dilution	Fever	Leukopenia	Illness	Deaths	
8	1	1×10^0	+++	+++	Mild	0	Immune
23	2	1×10^0	1/2 +++	2/2 +++	1/2 severe	0	Immune
24*	5	1×10^0	+++	+++	5/5 severe	4	0/1
29*	1	1×10^{-6}	—	—	—	—	1/1
29*	1	1×10^{-5}	—	—	—	—	1/1
29*	1	1×10^{-4}	+++	+++	1/1 severe	1	.....
29*	1	1×10^{-3}	+++	+++	1/1 severe	1	.....
29*	1	1×10^0	+++	+++	1/1 severe	1	.....

+++ = severe reaction.

* Grown in a 500-ml. Roux flask—total 50 cc. of culture.

dilution bottles. All 5 pigs inoculated with this virus, undiluted, showed severe temperature reactions and leukopenia. All showed severe signs of hog cholera and 4 of the 5 died. The fifth was immune to subsequent challenge. All of the following passages in this series were made in 500-ml. Roux flasks.

Passage 29 of the leukocyte series was titrated in swine (table 5). Dilutions up through 1×10^{-4} were positive in swine and caused severe illness and death. Higher dilutions produced no reactions and the inoculated pigs were subsequently susceptible to challenge with virulent virus.

DISCUSSION

The *in vitro* growth of the virus of hog cholera in the blood of swine offers new possibilities for the study of the pathogenesis of this lethal disease of swine. While the reticuloendothelial system has been shown to be directly involved in hog cholera infections,[15, 16] the role of the leukocyte has been generally assumed to be simply that of mechanical transportation of the infectious agent to susceptible cells. The experiments reported in this paper strongly indicate that leukocytes of the peripheral blood not only provide a transporting medium for the virus of hog cholera but, more importantly, furnish the necessary cellular requirements for its propagation.

There are many factors yet to be assessed in this manner of virus culture. The virus grows well in the presence of both erythrocytes and leukocytes, but appears to grow best when most of the erythrocytes are removed from the system. The effect of Tyrode's solution in different proportions was not explored. The incubation period in the experiments described was three days but the optimum period was not experimentally determined.

Two strains of virus grown under similar conditions responded differently with respect to pathogenicity and antigenicity. The Michigan A strain displayed altered pathogenicity and almost a complete lack of antigenicity in 22 passages. The temperature rise, leukopenia, and diarrhea were definite indications of a partially attenuated hog cholera virus. The lack of protective immunity, however, suggested that the virus was antigenetically impotent. This may be understandable since the original isolation of the virus was from a pig dying from hog cholera during a postvaccination infection in 1950 when hog cholera variants first were recognized.

The Sprankle virus responded to the *in vitro* blood culture passage by maintaining both pathogenicity and antigenicity in the leukocyte series. This indicated both a difference in the two virus strains and an advantage of the leukocyte culture over the whole blood culture.

The increased virulence associated with use of 500-ml. Roux flasks (table 5) may indicate either an advantage in using the larger quantity of culture or a more desirable gas-medium relationship.

The delayed serum technique offers a usable modification of the *in vivo* serum-neutralization test. The results obtained seemed to demonstrate clearly the existence of subprotective immunity. It further emphasizes that anti-hog cholera serum has little effect in highly susceptible swine which have been infected longer than three days.

SUMMARY

1) The hog cholera virus was shown to propagate in culture using cells from peripheral bloood.

2) Leukocytes but not erythrocytes appeared capable of supporting virus growth.

3) The alteration of a strain of hog cholera virus resulted from passage in blood cell culture but the resulting virus proved to be antigenetically impotent.

4) Two virus strains reacted somewhat differently in serial passage. The "Sprankle" strain appeared to be more readily adaptable to *in vitro* culture and showed less antigenic variation than the "Michigan A" virus.

5) A method was shown for the demonstration of subprotective immunity.

References

[1] Lewis, P. A., and Shope, R. E.: The Blood in Hog Cholera. J. Expti. Med., *50*, (1929): 719–736.

[2] Cahil, E. A.: Post Vaccination Trouble—A Possible Diagnostic Method. J.A.V.M.A., *27*, (1929): 425–431.

[3] Thorp, Frank, Jr., and Graham, Robert: A Study of the Leukocyte Changes in the Blood of Diseased Swine. J.A.V.M.A., *30*, (1930): 198–203.

[4] Cole, C. G.: Leukocyte Counts on the Blood of Normal, Cholera-Infected, and Recently Immunized Pigs. J.A.V.M.A., *34*, (1932): 392–400.

[5] Kernkamp, H. C. H.: The Blood Picture in Hog Cholera. J.A.V.M.A., *95*, (1939): 525–529.

[6] Shu, Shupei: Studies on the Cellular Changes in Pigs' Blood During the Development of Hog Cholera. Ann. Rep. New York State Vet. Coll., Cornell University (1929–1930): 124–136.

[7] Dinwiddie, R. R.: Studies on the Hematology of

Normal and Cholera-Infected Hogs. Univ. Arkansas Exper. Sta. Bull. 120, (1914): 1–41.

[8] Rucks, Willy, and Murray, Chas.: Infectivity of the Cells of Hog Cholera Blood. J.A.V.M.A., *78*, (1931): 691–702.

[9] Roderick, L. M., and Schalk, A. T.: Collected Studies on Hog Cholera. North Dakota Agr. . Coll. Exper. Sta. Bull. 210, (1927): 3–24.

[10] Traub, Erich: Studies on the *in Vitro* Multiplication of Newcastle Disease Virus in Chicken Blood. I. Virus Growth in Relation to Amount and Kind of Seed Virus, Time of Incubation and Number of Cells. Naval M. Res. Inst., Nat. Naval M. Center, Bethesda, Md., Project NM005 048.11.01, (1951): 1–14.

[11] Traub, Erich: Studies on the *in Vitro* Multiplication of Newcastle Disease Virus in Chicken Blood. II. Cultivation of the Virus in Leucocyte Suspension. Naval M. Res. Inst., Nat. Naval M. Center, Bethesda, Md., Project NM005 048.11.02, 1951.

[12] Traub, Erich: Studies on the *in Vitro* Multiplication of Newcastle Disease Virus in Chicken Blood. II. A Stabilizing Substance for Newcastle Disease Virus Present in Chicken and Mammalian Blood Cells. Naval M. Res. Inst., Nat. Naval M. Center, Bethesda, Md., Project NM005 048.11.03, (1951): 1–5.

[13] Dunne, H. W., Smith, E. M., Runnells, R. A., Stafseth, H. S., and Thorp, F., Jr.: A Study of an Encephalitic Strain of Hog Cholera Virus. Am. J. Vet. Res., *13*, (1952): 277–289.

[14] Parker, R. C.: Methods of Tissue Culture. Paul B. Hoeber, Inc., New York (1950): 77.

[15] Siefried, O., and Cain, C. B.: Histological Studies on Hog Cholera. II. Lesions of the Vascular System. J. Exptl. Med., *56*, (1932): 345–351.

[16] Bueno, P.: Alteracoes Cardiacas Encontradas em Casos de Peste Suina. Arq. Inst. Biol. Sao Paulo, *11*, (1949): 59–68.

The Use of Low Level Piperazine on Pigs Naturally Infected with *Ascaris Lumbricoides*

WILLIAM D. LINDQUIST, Sc.D.

East Lansing, Michigan

DURING JANUARY, 1956, an experiment was carried out with low level feeding of a piperazine (polymeric piperazine-1-carbodithioic acid)* to pigs experimentally infected with *Ascaris lumbricoides*. In that work, Lindquist [2] demonstrated that one tenth of a therapeutic dose of the drug per day in the feed was not sufficient to prevent either respiratory embarrassment or liver involvement. There were no ascarids in the intestines in any of the pigs. This absence of worms was explained by reference to the work of De Boer [1] and Martin [3] both of whom indicated, either by data or discussion, the difficulty of experimentally infecting pigs with ascarids.

Because of this failure to produce development of worms in the intestinal tract, the following experiment was devised using one fifth of a therapeutic dose per day in the feed and allowing the pigs to acquire Ascaris normally from infective pasture.

From the Department of Microbiology and Public Health, Michigan State University, East Lansing. Journal article 1992, Michigan Agricultural Experiment Station.

* Parvex® was supplied through the generosity of the Upjohn Co., Kalamazoo, Mich.

MATERIALS AND METHODS

Fifteen pigs, raised on screens soon after farrowing until introduced into this experiment, were used. Five different litters and two breeds were represented. They were about 8 weeks old at the initiation of the experiment.

A therapeutic dose was assumed to be 100 mg. per kilogram of body weight. With the pigs averaging approximately 16 kg., one fifth of this level would allow each pig 320 mg. per day of active piperazine. Considering 3 lb. of dry feed per pig per day, 107 mg. of active ingredient were incorporated per pound of feed.[2]

The pigs were divided into two groups at the beginning of the experiment and were kept in clean rooms with concrete floors. Ten animals in one group were fed the drug ration prior to removing 5 of them to the east pasture. These 5 "east pasture" pigs were continued on drug throughout the experiment. The 5 remaining animals received no further medication and served as negative controls. The 5 pigs in the other group were removed to the west pasture and kept as positive controls. One pig from each of the two pasture groups was killed at two, four, and six weeks after the pasture experiment started.

TABLE 1—Effect of Low Level Piperazine Feeding on Naturally Acquired Ascaris Infections in Pigs

Pig No.	Starting wt. (lb.)	Interval to necropsy (wk.)	Wt. at necropsy (lb.)	Wt. gain (lb.)	Larvae recovered at necropsy
		EAST PASTURE, MEDICATED FEED			
12–3	45	3	E59*	14	188
9–1	36	4	E54	18	11
9–10	32	5	69	37	2
13–7	38	5	89	51	3
13–8	36	6	106	70	85
Totals				190	289
		WEST PASTURE, NORMAL FEED			
12–5	31	3	E43	12	6
10–1	46	4	E69	23	47
38–8	36	5	90	54	86
38–6	38	5	92	54	6
38–1	40	6	107	67	4
Totals				210	149
		CONTROLS IN BUILDING			
12–4	43	3	E57	....	0
9–3	44	4	E63	....	0
38–7	41	5	91	....	0
12–6	42	6	108	....	0
9–4	34	6	93	....	0

* E = eviscerated weight.

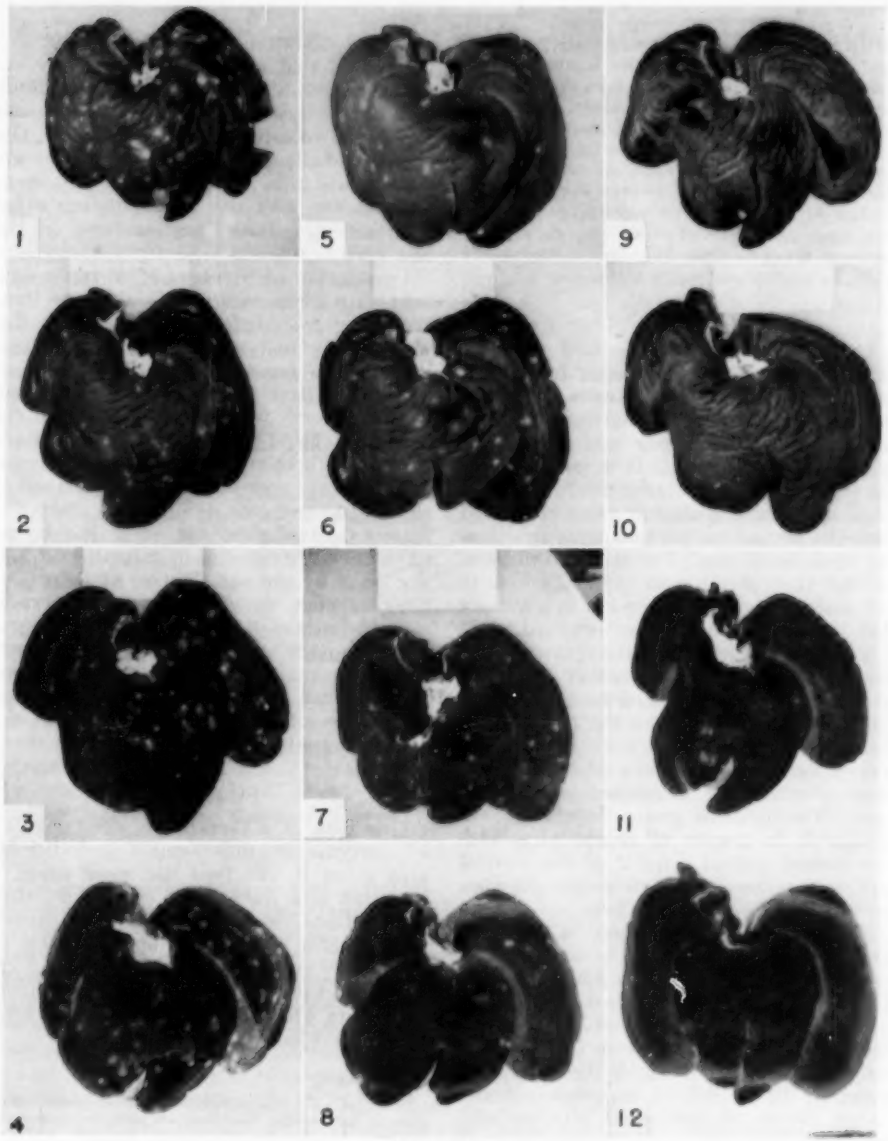


PLATE 1

Fig. 5 to 8—Livers from pigs from west pasture on normal feed. These pigs were killed at two, four, five, and six weeks, respectively, after the start of the experiment.

Fig. 5 to 8—Livers from pigs from west pasture on normal feed. These pigs were killed at two, four, five, and six weeks, respectively, after the start of the experiment.

Fig. 9 to 12—Livers from pigs on normal feed, raised on concrete, and killed at two, four, five, and six weeks, respectively, after the start of the experiment.

At the fifth week, necropsies were done on 2 pigs from each pasture. One negative control pig was slaughtered at the second, fourth, and fifth week intervals and the 2 remaining control pigs were killed with the six-week interval pasture pigs.

The contents of the small intestine of all pigs were screened through a combination of 40- and 60-mesh brass screens to recover developing ascarids. At the time of slaughter, photographs were made of the livers of all pigs to compare lesions of the three groups (plate 1). The results of the experiment are shown (table 1).

DISCUSSION

The level of piperazine used was not sufficient to act on the larvae present in the enteron. Since larvae were recovered at different intervals during their development, the amount of drug used may not be active on larval stages. It is possible that some larvae were removed while the pigs were on pasture, although when compared with the normal pasture controls this does not seem significant. There is also the possibility that if the host were allowed to live and the worms to develop to maturity that they would have been removed.

No feed intake measurements were undertaken but the drug ration appeared palatable. There was a difference in the total weight gain between the two pasture groups; however, there were not enough weight gain measurements taken to determine whether this difference was significant. The pasture groups could not be compared, in respect to weight gain, with the control group, since 2 of the control pigs were allowed to live to the six-week period.

The relationship of worm infection to liver scars has not often been discussed. In laboratory infected pigs, Lindquist [2] indicated that liver scars occurred in pigs without intestinal phases of the parasite being present, particularly in initial single infection animals. Plate 1 illustrates the livers of 12 of the 15 experimental animals. Livers of medicated pigs at two, four, five, and six weeks on pasture are shown (fig. 1 to 4). Livers of control pasture pigs are shown at the same weekly intervals (fig. 5 to 8). Livers of control pigs raised on concrete and slaughtered at the same intervals, respectively, are also shown (fig. 9 to 12). One or two spots were found on each control pig's liver but no worms were recovered in the intestinal screening.

On a basis of the liver illustrations, one might feel that the drug in some way encouraged production of liver scars. Such was not the case. Undoubtedly, the east pasture medicated group received the greatest challenge of worms, since for six years, until this year, east and west were one pasture with the pigs spending extra time near the feeder and waterer on the east end.

Correlation of numbers of worms recovered with liver scars is difficult. The liver from a pig on infective pasture for five weeks and from which only 2 intestinal worms were recovered (fig. 3) is comparable to the liver from a pig on pasture six weeks with 85 worms recovered from the intestine (fig. 4). Occasionally, one finds a pig with a clean liver and yet numerous worms. This may possibly be explained if the larvae get into lymphatics and completely bypass the liver or if the larvae have already migrated in a small host and are eaten by the pig. Tiner [4] and Sprent [5] demonstrated the development of certain ascarids in the final host without migration when the migratory phase was spent in an unusually small host. This apparently has not been demonstrated in the pig but may offer an explanation for occasional heavy infections without liver scarring.

SUMMARY

One fifth of a therapeutic dose per day of polymeric piperazine-1-carbodithioic acid given in the feed one week prior to infection and during the course of infection did not prevent migration of *Ascaris lumbricoides* larvae, nor was it capable of completely removing larvae from the 5 experimental pigs. One can not predict from these data what effect this continuous level would have on mature worms.

References

[1] De Boer, E.: Experimentelle Untersuchungen über *Ascaris lumbricoides* des Menchen und des Schweines. Ztschr. f. Infektionskr., *48*, (1935): 248–269.

[2] Lindquist, W. D.: The Use of Low Level Piperazine on Pigs Experimentally Infected with *Ascaris Lumbricoides*. Am. J. Vet. Res., *18*, (1957): 119–120.

[3] Martin, H. M.: Studies on the *Ascaris Lumbricoides*. Univ. Nebraska Agric. Exper. Sta. Res. Bull. 37, (Dec., 1926): 1–78.

[4] Tiner, J. D.: Preliminary Observations on the Life History of *Ascaris Columnaris*. J. Parasitol., (Sect. II, suppl.) *35*, (1949): 13.

[5] Sprent, J. F. A.: Intermediate Hosts in Ascaris Infections. J. Parasitol., (Sect. II, suppl.) *39*, (1953): 38.

Studies on the Pharmacology of Sulfaquinoxaline in Dairy Cattle

C. M. STOWE, V.M.D., Ph.D.; D. PALLESEN, D.V.M.; W. HARTMAN, D.V.M.

St. Paul, Minnesota

SULFAQUINOXALINE was synthesized in 1944 by Weijlard *et al.*,[1] and the chemistry of it and its derivatives was subsequently investigated by Stevens *et al.*[2] and Dux and Rosenblum.[3] The latter workers reported that the usual colorimetric method for determination of sulfonamides had to be modified for sulfaquinoxaline, since a nonlinear relationship exists between drug concentration and color intensity in the routine procedure.

Smith and Robinson [4] conducted *in vitro* tests with *Escherichia coli* and found that sulfaquinoxaline was about four times as effective as sulfanilamide at the same concentrations, but some 32 times less potent than sulfadiazine. On the other hand, in mice infected with standardized lethal doses of *Diplococcus pneumoniae*, sulfaquinoxaline was substantially more effective than either sulfathiazole or sulfadiazine. These results were explained by the fact that equivalent doses of sulfaquinoxaline maintained blood levels for a prolonged period of time (over 24 hours) as compared to sulfathiazole and sulfadiazine.[4] The striking difference between *in vitro* and *in vivo* potency points out the fallacies of predicting antibacterial potency of sulfonamides from *in vitro* studies alone.

Studies with rats showed that after intravenous administration, sulfaquinoxaline was secreted into, and retained in, the gastrointestinal tract.[5] This supposedly provided for continued absorption of the drug, thereby resulting in prolonged blood levels. Slow elimination also was stated to play a part in the prolongation of blood levels.[1] A slow decline in blood levels with detectable concentrations 112 hours following doses of 200 mg./kg. in poultry also was reported.[7]

Limited studies in cattle and sheep indicated an entirely different situation. These species do not seem to maintain high, prolonged levels following either oral or intravenous doses of sulfaquinoxaline.[8,9] Recent work shows that the drug disappears rapidly from the blood when given intravenously, and that with oral administration it is difficult to attain effective blood levels.[10] Once in the body, the drug is acetylated to a considerable extent.[10] In the urine of experimental animals, various metabolites, such as 2-sulfanilyl-3-hydroxy quinoxaline and acetylsulfaquinoxaline, as well as the unchanged drug, have been identified.[11] Sautter and Stowe [12] reported the presence of 3-hydroxy sulfaquinoxaline in the urine of cattle. The drug apparently is bound to a high extent by plasma proteins,[13] and it seems to penetrate the egg readily following oral treatment of poultry.[7]

Sulfaquinoxaline has found its greatest use in the treatment of poultry coccidiosis, although there have been a few reports of its use in bovine coccidiosis,[14,15] A few veterinarians have used the drug in the treatment of "foul foot" and certain systemic conditions in cattle. According to Eveleth *et al.*,[16] good results were obtained, with no toxic effects, in the treatment of swine pasteurellosis.

Toxic reactions to sulfaquinoxaline are both varied and serious. Jones *et al.*[9] reported muscle tremors, weakness, and falling following intravenous administration. Simesen[10] reported similar effects. Kidney damage was found in all cattle treated with sulfaquinoxaline[16]; other workers reported that kidney damage occurred in the rat and monkey when the plasma level exceeded 10 mg. per 100 ml. Other toxic effects that have been reported are a hypoprothrombinemia and prolonged prothrombin time.[4,6,17,18]

At the time this study was undertaken, the only available information on cattle was that of Jones *et al.*[9] In spite of these previous results, which were obtained in a small number of animals, the possibility remained, in view of the results in other species, that further studies with a larger number of cattle might reveal that the bovine species handled the drug in a manner

Paper 3555, scientific journal series, Minnesota Agricultural Experiment Station.

The work reported in this paper was supported in part by a grant from Sharp and Dohme Division of Merck and Co., West Point, Pa.

From the Division of Veterinary Physiology and Pharmacology, School of Veterinary Medicine, University of Minnesota, St. Paul (Stowe); practitioner, Sisseton, S. Dak. (Pallesen); and St. Paul, Minn. (Hartman).

TABLE 1—Sulfaquinoxaline Blood Levels in Calves—1.0 gr. per Pound Intravenously Initially, 1.0 gr. per Pound Orally at 24, 48, and 72 Hours

Time postadministration (hr.)	Milligrams per 100 ml. of whole blood								
	1065	1080	137	1123	1107	1124	1125	Av.	Range
0	0	0	0	0	0	0	0	.....	.....
1	17.1	14.2	11.7	14.0	16.2	23.0	30.0	18.0	11.7 to 30.0
2	12.4	8.0	8.9	14.1	14.7	13.9	21.0	13.3	8.0 to 21.0
4	7.0	2.4	6.1	13.3	8.1	10.7	16.0	9.1	2.4 to 16.0
6	2.7	0.4	3.8	11.7	3.7	3.8	10.6	5.3	0.4 to 11.7
9	0.7	0.3	5.8	8.3	0.6	2.4	....	3.0	0.3 to 8.3
12	0.2	0.2	1.8	6.2	0.3	1.4	9.5	2.8	0.2 to 9.5
24	0.2	0.2	0.9	1.9	2.4	0	5.2	1.5	0 to 5.2
36	2.4	0.3	1.2	1.4	1.0	....	....	1.2	0.3 to 2.4
48	2.4	1.6	0.4	0.6	0.6	0	3.3	1.3	0 to 2.4
60	1.9	1.0	1.5	1.0	1.0	....	3.5	1.7	1.0 to 3.5
72	0.8	0.6	2.3	1.5	0.2	0.3	2.8	1.2	0.2 to 2.8
84	.....	.....	.....	.....	.....	0.2	3.0	0.2	.....

similar to laboratory animals. If such were the case, the use of the drug as a long-acting sulfonamide might prove feasible.

EXPERIMENTAL PROCEDURE

Healthy Holstein-Friesian calves, heifers, and cows, ranging in weight from 100 to 1,400 lb., were used. After weighing the animals, appropriate oral or intravenous doses of the drug were given.* Prior to treatment, whole blood was withdrawn (50 ml.) and used for preparation of blood and plasma standards. After treatment, oxalated blood samples were obtained at regular intervals by jugular puncture.

All sulfonamide determinations were done in duplicate according to the method of Bratton and Marshall.[19] This method is applicable to milk, cerebrospinal fluid, blood, plasma, and urine (after appropriate dilution). Acetylation was determined after hydrolysis with hydrochloric acid, as previously described.[19] Experience in determining sulfaquinoxaline by this method soon revealed that the average recovery from whole blood and plasma was 72 and 85 per cent, respectively. To circumvent this problem, drug standards were prepared for each experiment in the particular body fluid being assayed. Standards for urine and cerebrospinal fluid were prepared in distilled water, since recovery in these cases was virtually 100 per cent.

Furthermore, at drug concentrations above 7.5 to 10.0 mg. per 100 ml., there is a lack of linearity between the logarithm of color intensity and concentration, while below these concentrations the Lambert-Bier law prevails. For this reason, samples for sulfaquinoxaline determination were diluted before assay so that the final concentration was within the linear portion of the standard curve.

Prothrombin time was determined at 12-hour intervals, using duplicate samples and the Quick method.[20] Urine was collected in the manner previously described by Stowe.[21] The apparent volume of distribution was determined by the method described by Marshall[22] and Fisher *et al.*[23]

* Soluble sulfaquinoxaline for parenteral use was prepared by dissolving the powder in 1 N sodium hydroxide and adjusting the pH to approximately 11.5 with 1 N HCl. Final drug concentrations were 2.0 gr. per milliliter (130.0 mg./ml.).

RESULTS

Whole Blood Levels Following Intravenous and Oral Administration.—The whole blood levels of sulfaquinoxaline in calves are shown (table 1) following an initial dose of 1.0 gr. per pound (143.0 mg./kg.) intravenously, and equivalent oral doses given 24, 48, and 72 hours later. Blood concentrations are shown for 5 calves (table 2) treated with 0.5 gr. per pound (71.0 mg./kg.) intravenously, and equivalent oral doses 24 and 48 hours later. Blood levels in cows are shown (table 3) following single doses of 0.5 gr. per pound intravenously. Data presented in these tables are graphically represented (fig. 1).

Plasma Concentrations Following Intravenous Administration.—For the plasma sulfaquinoxaline concentrations following

TABLE 2—Sulfaquinoxaline Blood Levels in Calves—0.5 gr. per Pound Intravenously Initially, 0.5 gr. per Pound Orally at 24 and 48 hours

Time post-administration (hr.)	Milligrams per 100 ml. of whole blood					
	1119	1124	1125	1134	1136	Av.
0	0	0	0	0	0	0
1	8.5	7.1	8.2	7.8	8.3	8.0
2	4.5	4.1	....	5.5	5.6	4.9
4	1.7	2.8	2.4	2.6	3.0	2.5
6	1.2	1.5	1.1	1.7	2.7	1.6
9	0.7	0.6	.....	0.7	2.2	1.1
12	0.5	0.5	0.5	0.8	1.3	0.7
24	0.2	0.4	0.3	0	0	0.2
36	0.9	....	0.3	0.2	0	0.4
48	0.8	1.5	0.3	0	0	0.5
60	1.6	0.6	2.2	1.7	0.1	1.2
72	1.4	0.5	2.0	0.5	0.3	0.9
84	1.3	1.8	0.4	0.6	0	0.8

TABLE 3—Sulfaquinoxaline Blood Levels in Cows—0.5 gr. per Pound (71 mg./kg.) Intravenously

Time postadministration (hr.)	Milligrams per 100 ml. of whole blood						
	Cow 1115	Cow 820	Cow 1074	Cow 1001	Cow 1032	Cow 1113	Av.
0	0	0	0	0	0	0	0
1	7.8	12.0	12.4	....	11.2	....	10.9
2	5.1	5.0	8.0	3.3	5.2	3.6	5.0
4	3.0	1.7	3.1	1.9	1.5	0.8	2.0
6	0.8	0.5	1.1	0.2	....	0.3	0.5
9	0.3	0	1.0	0	0.2	0.2	0.3
12	0.2	0	0.1	0.1	0	0.1	0.1
24	0	0	0.2	0	0	....	0.04

intravenous doses of 0.5 gr. per pound, the "average" reported (table 4) is an over-all average of cows and calves. Actually, the average values for calves were a little lower initially, but slightly higher from four to 12 hours. The difference is probably not significant.

Examination of the blood and plasma levels in tables 2, 3, and 4 shows that the blood levels are less than plasma levels. The average ratio between concentrations in blood and plasma is: blood = 0.72 plasma. At one hour after administration, the ratio is 0.85, decreasing to 0.62 at nine hours. This suggests that the drug penetrates erythrocytes initially but leaves the red blood cells more rapidly than it does plasma. In a sense, then, erythrocytes may initially serve as a reservoir, releasing the drug to the plasma over a period of several hours.

Apparent Volume of Distribution.—This can be calculated in two ways. The first method involves plotting the logarithm of plasma concentrations against time and extrapolating the resultant straight line to zero time. By dividing the value so obtained into the dose (mg./kg.), a calculation of the distribution in percentage of body weight can be made. The second method consists of calculating the distribution from the plasma concentration at one hour and dividing this value into the amount of drug remaining in the body at this time, *i. e.*, urine excretion for one hour must be deducted from the initial dose.

It must be pointed out that calculations of apparent volume of distribution involve a theoretical concept which is open to possible sources of error. These are: (1) no significant adsorption, or "fixation," to cell surfaces and (2) uniform distribution throughout various body fluids. Although these are admittedly rather broad assumptions, there is no other simple way of estimating or measuring tissue penetration of a drug. The alternate approach is to assay various tissues and body fluids directly.

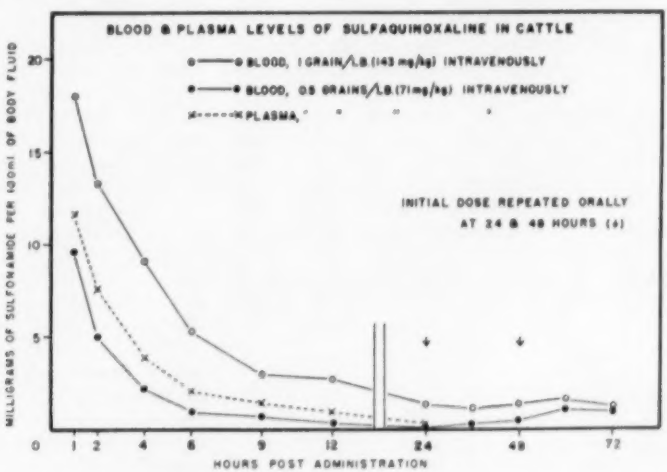


Fig. 1—Blood and plasma levels of sulfaquinoxaline in cattle.

TABLE 4—Plasma Levels of Sulfaquinoxaline in Cattle—0.5 gr. per Pound Intravenously (71 mg./kg.)

Time, post-administration (hr.)	Milligrams per 100 ml. of plasma																
	Calves									Cows							
	1119	1124	1125	1134	1136	1135	1138	1143	1144	1115	820	1074	1001	1032	1113	Av.	Range
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	.....
1	10.0	10.0	7.5	12.2	10.0	10.6	16.3	10.4	14.2	11.4	13.6	13.3	13.1	12.2	10.3	11.6	7.5 to 16.3
2	6.1	7.6	4.8	8.2	6.8	8.0	12.5	6.1	8.3	5.5	—*	8.7	9.0	8.1	6.0	7.6	5.5 to 12.5
4	2.5	3.6	—*	4.0	4.4	4.5	8.3	3.4	3.3	3.5	3.7	4.4	2.4	2.5	1.5	3.8	1.5 to 8.3
6	1.7	3.1	1.1	2.1	3.4	3.8	5.9	1.8	1.5	1.2	—*	1.4	—*	0.8	0.5	2.0	0.5 to 5.9
9	0.9	2.5	—*	0.8	1.9	2.5	4.4	—*	1.0	0.6	0.6	0.9	—*	0.6	0.2	1.4	0.2 to 4.4
12	—*	1.4	—*	0.3	1.3	1.0	2.7	0.7	0.5	—*	0.5	0.8	—*	0.5	—*	0.9	0.5 to 2.7
24	—*	0.2	—*	....	0.2	0.1	0.7	0.1	0	0.3	0.4	0.6	0	0.3	—*	0.2	0 to 0.7

* Not analyzed due to hemolysis in plasma sample.

The technical difficulties and potential sources of error implicit in such procedures leaves much to be desired. It is felt, therefore, despite the potential errors involved, that the volume distribution concept is worthy of application. In studies performed by Fisher *et al.*, good agreement was noted between actual tissue content of sulfonamides and that forecasted by the volume distribution concept.[23] The apparent volume of distribution shown (table 5) was obtained by both methods for 6 cows. All calculations are based on figures for total sulfonamide.

Plasma values obtained in calves enabled calculations to be made of the volume of distribution by the zero-hour method. An average value corresponding to 46 per cent of body weight was obtained. Although this is somewhat higher than the corresponding value for cows, it may be explained by the apparently greater amount of extracellular water in calves.[24] Substances such as sodium chloride, thiocyanate, and sulfisoxazole have an approximate volume of distribution of 20 to 30 per cent of body weight, indicating little or no cellular penetration. Sulfanilamide has a value of 87 to 98 per cent, while sulfathiazole, sulfadiazine, and sulfamerazine are distributed in a volume corresponding to 45 to 60 per cent of body weight.[23] Sulfaquinoxaline, with a value of 37 per cent (cows) to 46 per cent (calves), would appear to penetrate intracellular water to some extent.

TABLE 5—Apparent Volume of Distribution of Sulfaquinoxaline in Percentage of Body Weight

Cow No.	Calculated from:	
	0 time	1 hour
1074	35.2	39.0
1113	40.5	47.0
1001	36.6	38.3
1032	38.0	41.0
820	33.5	28.8
1115	39.1	43.2

Acetylation of Sulfaquinoxaline.—By acid hydrolysis of blood filtrates of animals treated with a sulfonamide, the degree of acetylation can be obtained. From the "total" sulfonamide obtained after hydrolysis, and the "free" sulfonamide obtained before hydrolysis, the percentage of acetylation can be calculated. In cows treated with sulfaquinoxaline intravenously, there appears to be an increasing degree of acetylation the longer the drug remains in the body (table 6). Acetylated sulfonamides are therapeutically inactive.

Milk Levels of Sulfaquinoxaline.—Composite milk samples from each quarter were taken concurrently with whole blood samples and assayed according to the standard method to determine the concentrations obtained in milk (table 7). It will be seen from these values that milk levels are only a fraction of the whole blood or plasma levels. This is in sharp contrast to certain other sulfonamides in which milk levels represent a significant fraction of blood levels.[25] Aside from this, the presence of sulfaquinoxaline in milk, even in low concentrations, indicates limited cellular penetration in the mammary gland.

Cerebrospinal Fluid Levels.—Cerebrospinal fluid samples were drawn from the foramen magnum of calves following intravenous administration. Table 8 shows the values obtained, along with the corresponding plasma levels. By a comparison of these values, it will be seen that cerebrospinal fluid concentrations are only 10 to 20 per cent of the plasma levels, indicating that in normal calves, at least, sulfaquinoxaline does not readily penetrate the spinal fluid. In the presence of inflamed me-

TABLE 6—Percentage of Acetylation in Blood Following Intravenous Doses of 1.0 gr. per Pound (143.0 mg./kg.)

Time postadministration (hr.)	Av. percentage of acetylation (6 cows)
1	19.9
2	22.0
4	29.5
6	36.0
9	48.0

ninges, a different situation may prevail, as in the case of sulfathiazole.

Urinary Excretion of Sulfaquinoxaline.—Kidney excretion of sulfaquinoxaline was determined by the method previously described.[21] The volume of urine excreted at any one time is measured and a sample is retained for the determination of sulfaquinoxaline. By continuing this process over a 24-hour period, the cumulative kidney excretion can be calculated. By analyzing the data so obtained for each cow, a composite graph of urine drug concentrations can be prepared (fig. 2). The total amount excreted in the free and acetylated form for a 24-hour period is shown for each cow (table 9). The average amount of the drug excreted in each form also is shown. Only about 50 per cent of the drug was excreted in the urine in the free and acetylated form. These determinations do not detect that portion of the drug, if any, that was excreted as the ethereal sulfate or glucuronide ester. In the case of these 6 cows, crystals were readily apparent in the urine samples from zero to six hours after dosing. These crystals were identified as 3-hydroxy-2 sulfanilamidoquinoxaline. This is the first time this metabolite has been detected in cattle on sulfaquinoxaline therapy and indicates that cattle are capable of oxidizing sulfonamides. The amount of this metabolite present in bovine urine was not determined but, from the appearance of the urine samples, it constituted a significant proportion of the total urine volume.

Toxicity of Sulfaquinoxaline.—When a 12.5 per cent sulfaquinoxaline solution is given intravenously at a rate approximating 40 to 100 ml. per minute, there frequently is seen a varying degree of ataxia, staggering gait, and partial posterior paralysis. In calves, these signs lasted for one to six hours, during which time the animals gradually returned to normal. In cows given a total dose of 0.5 gr. per pound (71.0 mg./kg.), these signs were accentuated; some of the animals "went down" for a period of one to three hours. Marked posterior weakness, arching of the back, staggering, and general depression also were observed in 5 of 7 cows. Gradual recovery took place over a period of 12 hours and, by the next day, all abnormalities had disappeared. The commonly used sulfonamides only infrequently produce such signs when given intravenously at comparable pH, concentration, dose, and rate of administration. The acute, toxic effects described above are similar to those reported by Jones *et al.*[9] No such abnormalities occurred following oral or intraperitoneal administration.

All cows given sulfaquinoxaline intravenously showed a marked degree of crystalluria from one to eight hours following administration. It is of interest that in figures 1 and 2 the appearance of crystals in the urine corresponded to the period of most rapid depletion of blood levels and the period of highest concentration of the drug in the urine. Despite this rather alarming crystalluria, no hematuria was seen. This indicates that the kidney damage, which in all likelihood was present, did not traumatize the kidney to the point of causing hemorrhage. Crystalluria was

TABLE 7—Milk Concentrations of Sulfaquinoxaline—0.5 gr. per Pound Intravenously (71.0 mg./kg.)

Time post-administration (hr.)	Milligrams per 100 ml. of milk*					
	1115	1074	1001	1032	1113	Av.
0	0	0	0	0	0	0
1	0.4(11.4)	....	0.6(13.1)	1.0(12.2)	8.8(10.3)	0.7
2	0.1(5.5)	1.3(8.3)	1.1(9.0)	1.1(8.1)	0.9(6.9)	0.9
4	0.2(3.5)	0.2(4.4)	0.8(2.4)	1.2(2.5)	0.3(1.5)	0.5
6	0.1(1.2)	0.2(1.4)	0.6	0.9(0.8)	0.3(0.5)	0.4
9	0 (0.6)	0.3(0.9)	0.3	0.4(0.6)	0.2(0.2)	0.2
12	0.2	0.2(0.8)	0.2	0.2(0.5)	0.2	0.2
19	0.1	0.3	0.3	0.1	0.1	0.2
24	0 (0.3)	0 (0.6)	0.4(0)	0.1(0.3)	0	0.1

* For purposes of comparison, the corresponding plasma levels are shown in parentheses.

TABLE 8—Cerebrospinal Fluid Concentrations in Calves—Intravenous Doses of 0.5 gr. per Pound and Equal Oral Doses Every 24 Hours

Time post-administration (hr.)	Milligrams per 100 ml. of cerebrospinal fluid*			
	1135	1138	1143	1144
0	0	0	0	0
1	1.8(10.6)	1.6(16.3)	....	....
2	....	....	0.7(6.1)	1.2(8.3)
4	0.8(4.5)	1.2(8.3)	....	....
6	....	....	0.5(1.8)	1.4(1.5)
72	0.3	0.04	1.2	0.3

* Corresponding plasma concentrations are shown in parentheses.

not observed following intravenous and intraperitoneal dosing of calves; it was likewise absent with oral dosing of calves and cows.

Several days following the adminstration of sulfaquinoxaline, the calves were killed and the kidneys were examined grossly and microscopically (table 10†). Descriptions of these kidneys were reported in another publication.[12] Simesen[10] reported similar findings in experimental studies with sulfaquinoxaline in cattle.

Attempts were made to evaluate the degree of kidney damage in calves by following blood urea nitrogen levels. Calves given the drug orally and intravenously over a period of several days showed no significant changes in these levels, despite the gross and microscopic lesions frequently found. However, it is known that blood urea nitrogen values are not a sensitive index of kidney function.

It has been shown previously that dogs treated with sulfaquinoxaline showed a hypoprothrombinemia that could be reversed by the administration of vitamin K.[17, 18] In view of this, plasma samples were taken in the appropriate manner over a period of several days and frozen. After all plasma samples had been collected, the prothrombin time was determined by the method of Quick.[20] The prothrombin time in calves gradually increased (table 11) from the pretreatment average of 18.1 seconds to 47.0 seconds at 60 hours, when doses of 1.0 gr. per pound were given orally every 24 hours for three days. It is evident that this is an average increase, and that each calf did not show prolonged prothrombin times. Cows given single oral or intravenous doses of 0.5 gr. per pound showed no significant changes in prothrombin time.

Summary and Conclusions

The results obtained from this study are in good agreement with those reported by Simesen,[10] as regards blood and plasma levels, except that we were unable to show the same distribution of drug between plasma and erythrocytes at concentrations above 2.5 mg. per 100 ml. In our experience, plasma concentrations were higher than those in whole blood (and, therefore, in erythrocytes) at all concentrations studied. No explanation is immediately ap-

† The authors are indebted to the Division of Veterinary Pathology and Parasitology for the examination of these tissues.

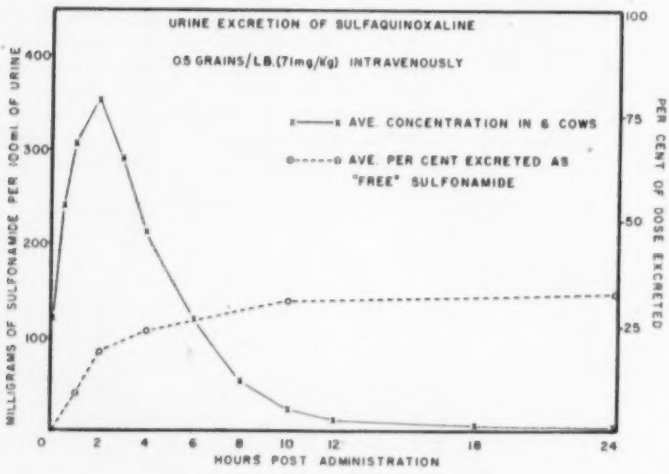


Fig. 2—Urine excretion of sulfaquinoxaline in cattle.

TABLE 9—Urinary Excretion of Sulfaquinoxaline in Cows—Intravenous Doses of 0.5 gr. per Pound (71.0 mg./kg.)

	Cow 1001	Cow 1074	Cow 820	Cow 1032	Cow 1115	Cow 1113	Av. excretion (% of dose)
Dose (Gm.)	37.4	38.8	43.4	40.1	33.0	40.0	.....
AMOUNT EXCRETED (GM. IN 24 HOURS)							
Free	9.7(25.9)*	10.5(26.9)	16.6(38.2)	12.8(32.0)	8.1(24.5)	11.9(29.7)	29.5(24.5 to 38.2)
Total	12.1(32.4)	23.3(60.0)	30.0(69.2)	19.0(47.0)	11.7(35.4)	18.6(46.5)	48.4(32.4 to 69.2)
Acetylated	2.4(6.4)	12.8(33.1)	13.4(31.0)	6.2(15.3)	3.6(10.6)	6.7(16.8)	18.9(6.4 to 33.1)

* Figures in parentheses indicate percentage of the dose excreted.

parent for this discrepancy. Simesen showed a high degree of binding to plasma proteins, despite a rapid disappearance of the drug from blood and plasma. These findings indicate that a high degree of protein binding, *per se*, does not significantly influence the duration of blood or plasma levels.

Several aspects of the pharmacology of sulfaquinoxaline in dairy cattle have been studied, including blood and plasma levels, acetylation, volume of distribution, urine excretion, and toxicity. On the basis of the work reported, sulfaquinoxaline appears to possess a number of undesirable properties. These are: (1) short duration of blood and plasma levels following intravenous administration, (2) low blood levels following oral administration, (3) rapid excretion through the kidney, and (4) serious toxic effects, including ataxia following intravenous administration, renal precipitation of the drug following both oral and intravenous administration, crystalluria, and interference with the blood-clotting mechanism. The fact that sulfaquinoxaline is a relatively insoluble sulfonamide (12.0 mg. per 100 ml. at pH 7.0),[26] coupled with its rapid rate of excretion, probably explains its toxic effect on the kidney.

TABLE 10—Toxic Effects of Sulfaquinoxaline

Animal	Total dose/day (gr./lb.)	Duration of treatment (da.)	Route of administration	Crystalluria*	Concretions in kidney on necropsy	Histological findings†
Calf 1065	1.0	4	i.v.‡ on day 1, oral on days 2, 3, 4	Not noted	+	+
137	1.0	4	i.v. on day 1, oral on days 2, 3, 4	Not noted	+	+
1117	1.0	4	i.v. on day 1, oral on days 2, 3, 4	Not noted	+	+
1080	1.0	4	i.v. on day 1, oral on days 2, 3, 4	Not noted	—	—
1124	1.0	3	i.v. on day 1, oral on days 2, 3, 4	.....	No necropsy	No necropsy
1124 (reused 3 wk. later)	0.5	3	Oral on days 2, 3, i.v. on day 1	Not noted	+	+
1125	1.0	4	i.v. on day 1, oral on days 2, 3	Not noted	No necropsy	No necropsy
1125 (reused)	0.5	4	i.v. on day 1, oral on days 2, 3	Not noted	+	+
1123	1.0	4	i.v. on day 1, oral on days 2, 3	Not noted	+	±
1119	1.0	4	i.v. on day 1, oral on days 2, 3	.....	—	—
1412	1.0	4	Oral only	Not noted	+	—
1414	0.5	4	Oral only	Not noted	—	—
1398	1.5	3	Oral only	Not noted	+	+
Cow 615§	0.5	2	Oral	Not noted	+	+
Cow 1115	0.5	1	i.v.	+	No necropsy	No necropsy
Cow 820	0.5	1	i.v.	+	No necropsy	No necropsy
Cow 1074	0.5	1	i.v.	+	No necropsy	No necropsy
Cow 1001	0.5	1	i.v.	+	No necropsy	No necropsy
Cow 1032	0.5	1	i.v.	+	No necropsy	No necropsy
Cow 1113	0.5	1	i.v.	+	No necropsy	No necropsy

* Crystalluria as used here refers to the appearance of crystals in the urine immediately after urination.

† Microscopic lesions consisted chiefly of crystals in the collecting tubules surrounded by inflammatory cells and areas of necrosis.

‡ i.v. = intravenous.

§ Four liters of blood (an estimated 10% of blood volume) were removed on each of the two days of treatment. The animal continued to eat and drink and appeared normal in all outward respects. Necropsy performed the following day.

TABLE 11—Prothrombin Time in Calves*

Hours	Route and calf No.											
	i.p.† 1123	i.v.† 1124	i.v. 1125	i.p. x137	i.v. 1107	i.v. x140	i.v. 1123	i.v. 1080	i.v. 1065	i.p. x140	i.v. x	Av.
0	19.0	15.0	17.0	21.0	.....	23.0	.....	18.5	15.0	16.5	21.0	18.1
12	29.0	24.0	24.0	21.0	15.0	26.5	28.0	18.0	28.0	22.0	22.0	23.4
24	38.0	20.0	.....	19.0	15.5	30.0	42.0	19.0	32.5	36.0	22.5	27.5
36	36.0	17.0	52.0	18.0	17.0	28.0	.....	17.0	20.5	44.0	.....	27.7
48	46.0	20.0	85.0	19.5	.....	31.0	98.5	18.0	19.0	50.0	.....	43.0
60	37.0	21.0	95.0	.....	20.0	48.0	108.0	17.0	19.0	57.0	.....	47.0
72	38.0	22.0	.....	19.5	19.0	66.0	93.5	17.0	18.0	40.0	.....	37.0
84	42.0	21.0	138.0‡	.....	.....	.....	.....	.....	.....	.....	.....	67.0‡

* Calves given 1.0 gr./lb. intravenously or intraperitoneally initially and 1.0 gr./lb. orally at 24 and 48 hours.

† i.p. = intraperitoneally and i.v. = intravenously.

‡ Average is high due to disproportionate weighing of calf 1125 at 84 hours.

From the pharmacological standpoint, two of the aims of sulfonamide therapy are: (1) the establishment and maintenance of therapeutic blood levels and (2) minimizing toxicity. In view of these criteria, sulfaquinoxaline appears to be a poor sulfonamide to select for systemic chemotherapy in dairy cattle. This would seem to be especially true in light of the more desirable properties of other sulfonamides, particularly sulfamethazine and sulfamerazine.

References

[1] Weijlard, J., Tishler, M., and Erickson, A.: Sulfaquinoxaline and Some Related Compounds. J. Am. Chem. Soc., *66*, (1944) : 1957–1959.

[2] Stevens, J., Pfister, K., and Wolf, F. J.: Substituted Sulfaquinoxalines. I. The Isolation and Synthesis of 3-Hydroxy-2-Sulfanilamido-Quinoxaline and of Related Quinoxalines. J. Am. Chem. Soc., *68*, (1946) : 1035–1039.

[3] Dux, J. P., and Rosenblum, C.: Spectrophotometric Determination of Sulfaquinoxaline. Anal. Chem., *21*, (1949) : 1524–1527.

[4] Smith, D. G., and Robinson, H. J.: Some Chemotherapeutic Properties of Sulfaquinoxaline. Proc Soc. Exptl. Biol. and Med., *57*, (1944) : 292–295.

[5] Silber, R. H., and Clark, I.: Factors Influencing the Maintenance of Blood Levels of Sulfonamides. Arch. Biochem., *10*, (1946) : 9–18.

[6] Seeler, A. O., Mushett, C. W., Graessle, O., and Silber R. H.: Pharmacological Studies on Sulfaquinoxaline. J. Pharm. and Exptl. Therap., *82*, (1944) : 357–363.

[7] Schlenker, F. S., and Simmons, B. K.: The Absorption, Distribution, and Excretion of Sulfaquinoxaline in Poultry. Am. J. Vet. Res., *11*, (1950) : 291–295.

[8] Cameron, H. S., and McOmie, W. A.: Absorption and Excretion of Sulfaquinoxaline in a Ruminant. Cornell Vet., *35*, (1945) : 47–51.

[9] Jones, L. M., Smith, H. A., and Roepke, M. H.: The Effects of Large Doses of Various Sulfonamides Injected Intravenously in Dairy Cattle. Am. J. Vet. Res., *10*, (1949) : 318–326.

[10] Simesen, B.: Sulfabenzpyrazin til Kvaeg. Nord. Vet.-med., *7*, (1955) : 625–640.

[11] Scudi, J. V., and Silber, R. H.: Urinary Excretion Products of Sulfaquinoxaline. J. Biol. Chem., *156*, (1944) : 343–348.

[12] Sautter, J., and Stowe, C. M.: Symposium on Poisoning: Sulfonamide Intoxication Experimentally Produced in a Heifer. J.A.V.M.A., *130*, (1957) : 20–22.

[13] Bankowski, R. A., and Johnson, R. E.: The Binding of Sulfonamide Drugs to the Plasma Proteins of Chicken Blood. Am. J. Vet. Res., *10*, (1949) : 282–283.

[14] Dunlap, R. E.: Bovine Coccidiosis. Southwest. Vet., *2*, (1949) : 44–45.

[15] Frank, E. R.: Coccidiosis in Beef Cattle. North Am. Vet., *30*, (1949) : 238–239.

[16] Eveleth, D. F., Goldsby, A. I., and Bolin, F. M.: Sulfaquinoxaline in Field Cases of Pasteurellosis of Swine. Vet. Med., *44*, (1949) : 304–305.

[17] Mushett, C. W., and Seeler, A. O.: Hypoprothrombinemia Resulting from the Administration of Sulfaquinoxaline. J. Pharm. and Exptl. Therap., *91*, (1947) : 84–91.

[18] Eads, F. E., and Sholl, L. B.: Blood Studies in Dogs Following the Administration of Sulfaquinoxaline. Michigan State Coll. Vet., *10*, (1950) : 110.

[19] Bratton, A. C., and Marshall, E. K.: A New Coupling Component for Sulfanilamide Determination. J. Biol. Chem., *128*, (1939) : 537–550.

[20] Quick, A. J.: The Prothrombin in Hemophilia and in Obstructive Jaundice. J. Biol. Chem., *109*, 1935.

[21] Stowe, C. M.: Sulfisoxazole—Some Aspects of Its Pharmacology in Dairy Cattle. J.A.V.M.A., *123*, (1953) : 315–318.

[22] Marshall, E. K.: Distribution of 3,4-Dimethyl 5-Sulfanilamidoisoxazole in the Body. Proc. Soc. Exptl. Biol. and Med., *86*, (1948) : 472–473.

[23] Fisher, S. H., Troast, L., Waterhouse, A., and Shannon, J. A.: The Relation Between Chemical Structure and Physiological Disposition of a Series of Substances Allied to Sulfanilamide. J. Pharm. and Exptl. Therap., *79*, (1943) : 373–391.

[24] Inglis, J., Jennings, F., Lauder, I., Mulligan, W., and Weipers, M.: The Volume Distribution of Intravenously Injected Na^{24} in Bovines. Vet. Rec., *67*, (1955) : 765–768.

[25] Maclay, M., and Slavin, G.: Sulphonamide Investigations. Vet. Rec., *59*, (1947) : 313–317.

[26] Jones, L. M.: Veterinary Pharmacology and Therapeutics. Iowa State College Press, Ames, 1954.

The Metabolism of Phenothiazine, N. F., in Dairy Calves

I. Chronological Distribution of Drug in Digestive Tract Contents

T. ELLISON, M.S., and A. C. TODD, Ph.D.

Madison, Wisconsin

THE USE of phenothiazine as an anthelmintic in dairy cattle largely has been limited to treatment of young animals. This limited use of phenothiazine has been based upon its reported possible appearance in cows' milk and upon presumed toxicity of the drug.

The literature has many conflicting reports on the nature of the pharmacological action of phenothiazine. Sensitization reactions to phenothiazine, N.F., by cattle have been recorded by Clare *et al.*,[1,2] Duthby and Lewis,[3] and Whitten *et al.*[4-7] Limited information has been published on the manner and location in which phenothiazine was modified in the ruminant body.

The present paper summarizes the work concerned with the chronological distribution of phenothiazine and its derivatives in digestive tract contents of dairy calves. Other phases of the investigations will be presented in subsequent publications.

MATERIALS AND METHODS

Eighteen grade dairy calves ranging in weight from 150 to 525 lb. were used in this study. These calves had been on roughages prior to their receipt at this laboratory. The laboratory ration consisted of 2 lb. of ground grain per day and a good-quality alfalfa and grass hay offered *ad libitum*. The ground grain consisted of corn, oats, bran, linseed oil meal, and iodized salt.

The calves were each dosed with 101.3 Gm. of drench-grade phenothiazine (100.0 Gm. of phenothiazine, N.F.) in 6 oz. of warm, distilled water followed by a rinse of 2 oz. of water. Lecithin was used as the wetting agent in the drench-grade phenothiazine. Average particle size of the phenothiazine, N.F., was 10 to 15 μ.[8]

Each animal was slaughtered at a designated hour after drug administration. An incision was made on the median line of the ventral body wall of the calf from the anus to the trachea. Sections of the digestive tract to be analyzed were tied off. The gallbladder was severed at the point of entrance of the bile duct into the duodenum. Portions of the duodenum were designated as (a) "proximal duodenum," from the pylorus to the bile duct entrance, and (b) "distal duodenum," from the bile duct entrance to a point 2 feet posterior. After the duodenum was removed, the rectum was tied off close to the anus.

To arbitrarily standardize the division of the remaining small intestine into its various components, the first 12 feet posterior to the end of the duodenum were designated as the "jejunum." That portion of the intestine 12 feet anterior to the ileocecal valve was designated as the "ileum." The remaining small intestine was designated the "mixed jejunum-ileum."

The various compartments of the stomach and the colon, cecum, and rectum were isolated. All contents were removed from the digestive tract, weighed, and representative aliquots removed for analysis.

The digestive tract contents were dried in a hot-air oven at 120° C. for 48 hours to determine the percentage of moisture. The contents were then ground and 1.0-Gm. aliquots were extracted with 25.0 ml. of boiling water added in three successive extractions and the extracts were filtered on Whatman No. 1 filter paper. The aqueous extractions were diluted to 25.0 ml. with distilled water. The contents were then extracted similarly with 25.0 ml. of boiling 95 per cent ethanol.

Alcoholic extractions contained phenothiazine in the unchanged form. Aqueous extractions contained the water-soluble, oxygenated derivatives of phenothiazine, *i.e.*, phenothiazone, leukophenothiazone, thionol, leukothionol, and their conjugates.

The estimation of phenothiazine was based on methods devised by DeEds and Eddy[9] and modified by Cupples.[10] Aliquots of 0.1 ml. of the alcoholic extracts were pipetted into Evelyn colorimeter tubes and treated with 0.5 ml. saturated bromine water and 8.5 ml. of 95 per cent ethanol. The tubes were then placed in a hot water bath to develop the color of the reaction and remove excess bromine. Colorimetric determinations were made with a 520-mμ filter in an Evelyn photoelectric colorimeter.

The addition of 9.9 ml. of concentrated hydrochloric acid to 0.1 ml. of the aqueous extractions developed the characteristic red color of phenothiazone. Determinations were made at 520 mμ.

From the Department of Veterinary Science, University of Wisconsin, Madison. Papers N.S. 225-228, published with the approval of the director of the Wisconsin Agricultural Experiment Station and supported in part by E. I. DuPont DeNemours and Co. and by a grant from the Research Committee, Graduate School, University of Wisconsin, with funds provided by the Wisconsin Alumni Research Foundation.

Standard curves were prepared from synthesized purified phenothiazone and repurified phenothiazine mixed in digestive tract contents of a control calf.

RESULTS

Chronological distribution of the drug in digestive tract contents was established by selective slaughter of 18 dairy calves at definite time intervals after administration of the drug. The time intervals selected were 1, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 40, 80, and 120 hours post-treatment.

Quantitative data obtained at each period are detailed in tables 1 to 3, which summarize the three major portions of the ruminant digestive tract.

Stomach.—Chronological distribution of the drug is shown (table 1). At one hour post-treatment, 89.8 Gm. of drug was recovered as phenothiazine in the rumen; 2.6 Gm. of the drug was recovered as phenothiazone. Phenothiazine and its principal oxygenated derivative were present in all four compartments of the stomach at one hour post-treatment.

The phenothiazine level in the omasum and reticulum increased sharply after two hours; the omasum contained 28.7 Gm. of phenothiazine and little oxidation had occurred in the reticulum and omasum. The drug levels of the abomasum remained relatively unchanged until after eight hours. Between three and six hours post-treatment, the phenothiazine level of the omasum remained high; the levels of the drug in the rumen rapidly decreased. In the omasum and abomasum, at eight hours, large amounts of phenothiazine were found but no correspondingly large increase in the phenothiazone level. The peak level of phenothiazine occurred in the abomasum at 12 hours post-treatment. The oxidation of phenothiazine occurred primarily in the rumen. The reticulum, omasum, and abomasum did not contain large amounts of the oxidized derivatives, even though large quantities of the unchanged drug were present.

After 12 hours post-treatment, the drug moved rapidly out of the rumen; the greatest decrease was between 22 and 24 hours. The omasum continued to concentrate large quantities of the drug and, at 24 hours, the phenothiazine content nearly equaled that of the rumen.

No trace of phenothiazine was present in the stomach at 80 hours post-treatment; phenothiazone was detected in small traces. No drug could be detected in the contents of the stomach at 120 hours.

Small Intestine.—The chronological distribution of drug is shown (table 2).

The first appearance of phenothiazine, but no trace of phenothiazone, was detected in the proximal duodenum at one hour post-treatment.

The greater solubility of the phenothiazone over phenothiazine was apparent in its more rapid distribution through the small intestine. Phenothiazine was not detected posterior to the jejunum at the end of two hours but phenothiazone had moved as far as the ileum.

At three hours post-treatment, both phe-

TABLE 1—Chronological Distribution of Drug in Stomach Contents from Dairy Calves Administered 100 Gm. of Phenothiazine, N.F. (Drench Grade)

Hours post-treatment	Rumen		Reticulum		Omasum		Abomasum	
	P-zine*	P-zone†	P-zine	P-zone	P-zine	P-zone	P-zine	P-zone
1	89.8483	2.6228	1.207	0.0696	0.4007	0.0481	0.0212	0.004
2	52.4111	1.7611	7.8348	0.0881	28.7753	0.0741	0.0525	0.0122
3	50.9586	11.2278	0.5963	0.2236	17.8354	2.0579	0.7168	0.0779
4	54.607	8.8552	2.3885	0.2961	10.717	0.8038	0.8987	0.2022
5	31.9116	7.8006	1.371	0.1567	16.9425	1.3554	0.2538	0.1088
6	31.6127	7.9032	1.0972	0.2438	12.1709	1.5374	0.2512	0.0594
8	29.6676	2.8343	0.717	0.0346	19.7655	0.2965	5.8863	0.0224
10	31.0027	9.046	1.2845	0.2977	3.9157	0.3537	2.9788	0.0441
12	44.7572	2.2379	1.3898	0.1069	2.3028	0.1187	16.0632	0.0996
14	40.2476	3.9028	0.8429	0.1124	6.6444	0.3854	5.9598	0.0488
16	33.8946	1.2498	0.204	0.0644	6.0047	0.1729	0.4118	0.0238
18	33.5582	4.382	0.3734	0.0636	15.9725	0.489	3.6326	0.1759
20	27.2291	5.4458	0.4105	0.0821	8.4863	0.6457	8.1406	0.3624
22	24.2765	6.0691	1.256	0.1365	13.3803	1.1894	1.0676	0.0234
24	8.9552	6.7164	0.3309	0.2316	8.1136	3.0426	1.3256	0.12
40	6.1888	2.3208	0.2969	0.1113	6.2934	1.3845	0.733	0.0312
80	0	0.4558	0	0.094	0	0.3024	0	0.0109
120	0	0	0	0	0	0	0	0

* Unchanged phenothiazine (total grams of drug). † Phenothiazone and other water-soluble, oxygenated phenothiazine derivatives (total grams of drug).

TABLE 2—Chronological Distribution of Drug in Small Intestine Contents from Dairy Calves Administered 100 Gm. of Phenothiazine, N.F. (Drench Grade)

Hours post-treatment	Proximal duodenum		Distal duodenum		Jejunum		Mixed jejunum-ileum		Ileum	
	P-zine*	P-zone†	P-zine	P-zone	P-zine	P-zone	P-zine	P-zone	P-zine	P-zone
1	0.0015	0	0	0	0	0	0	0	0	0
2	0.0141	0.0003	0.0065	0.0076	0.0057	0.0145	0	0.0139	0	0.0083
3	0.0228	0.0013	0.0086	0.0036	0.1138	0.0253	0.2086	0.3911	0.0474	0.0355
4	0.0186	0.0047	0.003	0.0069	0.0045	0.0224	0.1146	0.1994	0.2467	0.1371
5	0.2113	0.019	0.0037	0.0054	0.0074	0.026	0.0144	0.156	0.0502	0.0376
6	0.0116	0.0009	0.0036	0.0023	0.0998	0.0665	0.0496	0.1092	0.0133	0.0267
8	0.0718	0.0012	0.0094	0.0113	0.0217	0.0507	0.5835	0.1089	0.2034	0.0324
10	0.1341	0.0033	0.096	0.0417	0.0848	0.0381	0.8685	0.1158	0.7533	0.2184
12	0.0031	0.0013	0.0007	0.0013	0.2139	0.01	0.6129	0.0629	1.274	0.0392
14	0.099	0.0004	0.0018	0.0032	1.0458	0.0837	1.1196	0.1796	2.157	0.2229
16	0.0025	0.0002	0.0092	0.0264	0.0563	0.017	0.4809	0.1154	0.3699	0.0308
18	0.0231	0.0048	0.019	0.0134	0.0941	0.0359	0.4546	0.0687	0.2453	0.069
20	0.0012	0.0002	0.035	0.0114	0.0149	0.0158	1.7489	0.6296	3.7223	0.072
22	0.1093	0.0086	0.1067	0.0406	0.19	0.1641	0.1958	0.1762	0.5665	0.4358
24	0.3068	0.0011	0.213	0.0054	0.203	0.0706	1.1329	0.3213	0.755	0.4797
40	0.0315	0.0011	0.0044	0.0179	0.1006	0.0503	0.2751	0.3312	0.2399	0.3599
80	0	0	0	0	0	0	0	0.0358	0	0
120	0	0	0	0	0	0	0	0	0	0

* Unchanged phenothiazine (total grams of drug). † Phenothiazone and other water-soluble, oxygenated phenothiazine derivatives (total grams of drug).

nothiazine and phenothiazone were detected in the ileum. The total concentration of phenothiazone at three hours nearly equaled that of phenothiazine.

At five hours, the phenothiazone concentration in the small intestine posterior to the bile duct was three times the concentration of phenothiazine.

The ratio of phenothiazine:phenothiazone was nearly 1:1 in the small intestine at 22 hours. At 80 hours post-treatment, phenothiazone was detected in the mixed jejunum-ileum but there were no traces of phenothiazine. No traces of drug were present in the small intestine at 120 hours.

Large Intestine.—The chronological distribution of the drug is shown (table 3).

Phenothiazine and phenothiazone first appeared in the colon and cecum at eight hours post-treatment. The first appearance of the drug in the feces (rectum) occurred at ten hours. The colon concentrated most of the drug present in the large intestine; the peak concentration of phenothiazine was 12.7612 Gm. at 20 hours.

The ratio of phenothiazine:phenothiazone at 22 hours post-treatment was nearly 1:1 in the rectum and 2:1 in the colon and cecum. At 80 hours, only traces of phenothiazone remained in the colon. No traces of the drug were detected in the large intestine at 120 hours.

DISCUSSION

The appearance of phenothiazone in the ruminant stomach within one hour post-treatment indicated that considerable oxidation of the phenothiazine does occur within this organ. That the primary site of oxidation of phenothiazine was the rumen is evident from the high levels of phenothiazone at all time intervals through 40 hours in this organ. Such comparatively high ratios of phenothiazone:phenothiazine were not detected in the reticulum, omasum, and abomasum.

Oxidation of the phenothiazine within the calf rumen is not necessarily evidence of a high oxygen tension within this organ. The source of such an oxygen tension is

TABLE 3—Chronological Distribution of Drug in Large Intestine Contents from Dairy Calves Administered 100 Gm. of Phenothiazine, N.F. (Drench Grade)

Hours post-treatment	Colon		Cecum		Rectum	
	P-zine*	P-zone†	P-zine	P-zone	P-zine	P-zone
1	0	0	0	0	0	0
2	0	0	0	0	0	0
3	0	0	0	0	0	0
4	0	0	0	0	0	0
5	0	0	0	0	0	0
6	0	0	0	0	0	0
8	0.1168	0.0467	0.8138	0.1411	0	0
10	1.3821	0.6019	1.0539	0.2235	0.5812	0.313
12	0.6274	0.1525	2.985	0.2985	0.0253	0.0101
14	3.151	0.3151	2.1808	0.2088	0.1559	0.085
16	0.8809	0.1019	1.4767	0.1424	0.5004	0.2602
18	0.8733	0.2382	0.6885	0.241	0.3128	0.1155
20	12.7612	0.5982	2.1832	0.2287	0.3949	0.0592
22	0.4778	0.2184	0.607	0.2732	0.2298	0.2043
24	2.6007	1.2385	0.5515	0.3237	0.402	0.268
40	1.1288	0.8708	0.33	0.1941	0.3606	0.2193
80	0	0.0388	0	0	0	0
120	0	0	0	0	0	0

* Unchanged phenothiazine (total grams of drug). † Phenothiazone and other water-soluble, oxygenated phenothiazine derivatives (total grams of drug).

not within the scope of this investigation.

The progression of the drug through the ruminant digestive tract is not constant. Primary sites of phenothiazine concentration are the rumen, the omasum, and the colon. Phenothiazine, present in all portions of the digestive tract at 40 hours post-treatment, had been eliminated completely by 80 hours.

Phenothiazone, the principal derivative, persists within the calf after the elimination of phenothiazine. Within the digestive tract, high levels of phenothiazone persist within the stomach, *i.e.*, the rumen and the omasum.

The more rapid movement of phenothiazone into the small intestine, in comparison with phenothiazine, evidently is related to its solubility but perhaps also to rumen absorption and delivery in bile.

At 120 hours post-treatment, no traces of this drug were found in the digestive tract.

SUMMARY

1) The methods of analysis of phenothiazine and its derivatives in the digestive tract contents of calves is described.

2) The first appearance of phenothiazone occurred in the rumen within one hour post-treatment; smaller amounts were also present in the reticulum, omasum, and abomasum.

3) No drug was detected in the digestive tract posterior to the proximal duodenum in one hour post-treatment.

4) First detection of phenothiazine in the feces occurred at ten hours.

5) At 22 hours post-treatment the ratio of phenothiazine to phenothiazone was nearly 1:1 in the jejunum, jejunum-ileum, ileum, and rectum, and a 2:1 ratio was detected in the colon and cecum.

6) The concentration of phenothiazine was greater in the omasum than rumen at 40 hours.

7) At 80 hours post-treatment, no traces of phenothiazine were detected in the digestive tract but phenothiazone (0.9377 Gm.) was detected in the rumen, reticulum, omasum, abomasum, mixed jejunum-ileum, and colon. No trace of phenothiazone was detected in the proximal and distal duodenum, jejunum, ileum, cecum, and rectum.

8) No traces of phenothiazine or phenothiazone were detected in digestive tract contents at 120 hours post-treatment.

References

[1] Clare, N. T.: A Photosensitized Keratitis in Young Cattle Following the Use of Phenothiazine as an Anthelmintic. II. The Metabolism of Phenothiazine in Ruminants. Austral. Vet. J., *23*, (1947): 340–344.

[2] Clare, N. T., Whitten, L. K., and Filmer, D. B.: A Photosensitized Keratitis in Young Cattle Following the Use of Phenothiazine as an Anthelmintic. III. Identification of the Photosensitizing Agent. Austral. Vet. J., *23*, (1947): 344–348.

[3] Duthby, B. L., and Lewis, R. W. E.: Eye Trouble Attributable to Photosensitization Following the Use of Phenothiazine in Bovines. East Afric. Agric. J., *14*, (1949): 155–156.

[4] Whitten, L. K., Clare, N. T., and Filmer, D. B.: A Photosensitized Keratitis in Cattle Dosed with Phenothiazine. Nature, *157*, (1946): 232.

[5] Whitten, L. K.: Calves Temporarily Blind After Phenothiazine Drenching. New Zealand J. Agric., *74*, (1947): 179.

[6] Whitten, L. K., and Filmer, D. B.: A Photosensitized Keratitis in Young Cattle Following the Use of Phenothiazine as an Anthelmintic. I. A Clinical Description with a Note on Its Widespread Occurrence in New Zealand. Austral. Vet. J., *23*, (1947): 336–340.

[7] Whitten, L. K.: Photosensitized Keratitis in Calves After Dosing with Phenothiazine. Internat. Vet. Cong. Rep., *14*, (1949): 56–60.

[8] Clover Chemical Co., Pittsburgh, Pa.: Personal communication.

[9] DeEds, F., and Eddy, C. W.: Studies on Phenothiazine. I. A Colorimetric Method for Estimation of Phenothiazine. Food Res., *2*, (1937): 305–309.

[10] Cupples, H. L.: Colorimetric Determination of Phenothiazine. Indust. and Engin. Chem., *14*, (1942): 53.

II. Chronological Levels of Drug in Blood Serum, Bile, and Urine

The materials to be presented here are concerned with the chronological appearance of phenothiazine and its derivatives in the blood serum, bile, and urine following treatment of 18 grade dairy calves. The appearance of the drug and its derivatives outside the digestive tract is of interest in view of widespread acceptance of phenothiazine therapy in livestock practice. A more complete understanding of the pharmacology of phenothiazine is of particular value in veterinary practice related to the dairy industry.

The oxidation products of phenothiazine which appear in the urine have been the subject of several reviews. Clare [1] and Harpur *et al.*[2] concluded that the urine of sheep and cattle contained mostly the potassium ethereal sulfate of leukophenothiazone, together with unconjugated leukophenothiazone and leukothionol and a conjugate yielding phenothiazine on acidification. Conjugated leukophenothiazone has been reported in the blood of sheep by Swales and Collier.[3]

MATERIALS AND METHODS

This study involved a continuation of the ob-

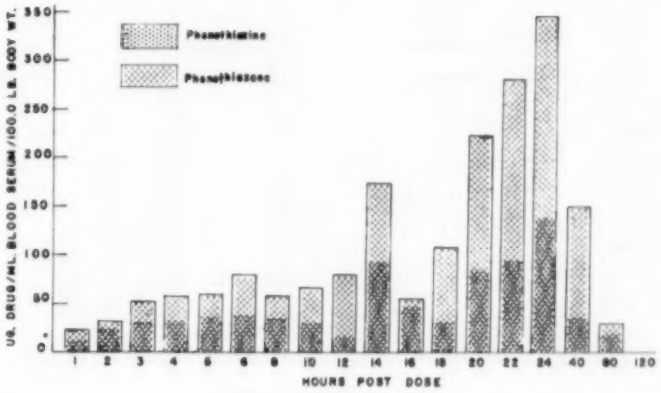


Fig. 1—Blood serum chronological levels of phenothiazine and phenothiazone in dairy calves.

servations made on 18 grade dairy calves weighing 150 to 525 lb. A urine sample was obtained from each animal prior to slaughtering. The calves were dosed, stunned at the designated hour post-treatment, the jugular vein was cut, and blood samples were collected for analysis. The gallbladder was tied off and the bile removed.

After coagulation, the blood serum was removed and centrifuged at 2,000 r.p.m. for 15 minutes. To analyze for unchanged phenothiazine, 1.0 ml. of blood serum was pipetted into a 15.0-ml. tube and mixed thoroughly with 8.5 ml. of 95 per cent ethanol. The tubes were then centrifuged at 2,000 r.p.m. for ten minutes. The supernatant was decanted into an Evelyn colorimeter tube (18 by 150 mm.), 0.5 ml. of saturated bromine water was added, and the tubes were placed in a hot water bath for five minutes. The contents were diluted to 10.0 ml. with 95 per cent ethanol and colorimetric determinations were made with a 520-mμ filter in the Evelyn photoelectric colorimeter.

In the analysis of phenothiazone, the protein in blood serum was precipitated with concentrated hydrochloric acid. Excess concentrated hydrochloric acid was added to redissolve the precipitated protein. Aliquots of 1.0 ml. of blood serum and 9.0 ml. of concentrated hydrochloric acid were added to each colorimeter tube and the determinations were made with a 520-mμ filter.

It was impossible in these studies to determine the exact amount of drug present in the circulatory system; no methods were provided to determine the total blood volume of each animal. Standard curves were prepared from blood serum obtained from a control animal.

Bile analysis was accomplished with dilution of the bile with 95 per cent ethanol or distilled water. Five milliliters of bile were pipetted into a 25.0-ml. volumetric flask and diluted to volume with 95 per cent ethanol. Aliquots of 1.0 ml. were removed for analysis of phenothiazine by bromination. To each colorimeter tube was added 0.5 ml. of saturated bromine water and 8.5 ml. of 95 per cent ethanol. The tubes were placed in a hot water bath for five minutes and read at 520 mμ. Five milliliters of bile were diluted to 25.0 ml. with distilled water for analysis of water-soluble phenothiazine derivatives. Aliquots of 1.0 ml. of aqueous bile and 9.0 ml. of concentrated hydrochloric acid were added to each colorimeter tube. Determinations were made at 520 mμ.

Analysis of urine was similar to the analysis of bile. The urine samples were placed in an oven at 70 C. for several hours to dissolve any bovine urinary conjugate of phenothiazine that may have precipitated. Two 1.0-ml. aliquots of each urine sample were pipetted into 100.0-ml. volumetric flasks. One flask was made to volume with distilled water and the other with 95 per cent ethanol. Aliquots of 4.0 ml. of the alcohol dilutions, 0.5 ml. of saturated bromine water, and 5.5 ml. of 95 per cent ethanol were added to each colorimeter tube. To determine phenothiazone, 4.0 ml. of aqueous urine and 6.0 ml. of concentrated hydrochloric acid were added to each colorimeter tube and determinations were made at 520 mμ. Total volume urine elimination from each animal was not determined.

The water-soluble, oxygenated derivatives of phenothiazine have been grouped under the name phenothiazone, the major component. Other derivatives present in trace amounts in the samples analyzed were leukophenothiazone, thionol, leukothionol, and the conjugates of these derivatives.

RESULTS

Blood Serum.—Chronological levels of phenothiazine and phenothiazone are shown (fig. 1 and 2). Within one hour after dosing, phenothiazine and phenothiazone were present in blood serum. The rapid oxidation of phenothiazine in the rumen evidently gave rise to the large amount of the oxygenated derivative in the blood serum. The presence of the unchanged phenothiazine in the blood was noteworthy at this time interval because

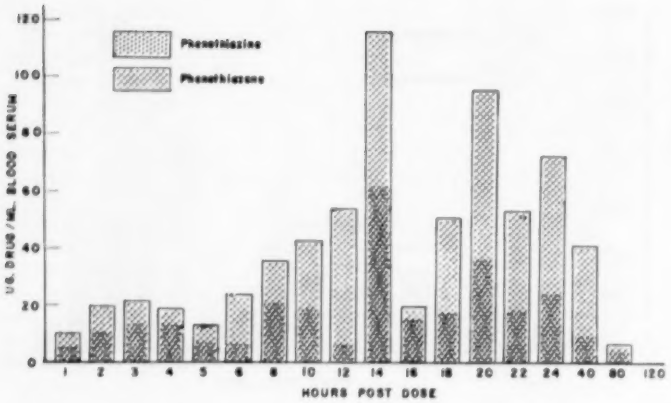


Fig. 2—Blood serum chronological levels of phenothiazine and phenothiazone in dairy calves.

of the known great insolubility of the drug.

To properly evaluate the drug levels in the blood serum, a weight relation factor of each animal should be considered. Since no blood volume data were available, the conversion of the drug level to a figure per 100 lb. of live body weight produced a more uniform picture (fig. 1). There were considerable fluctuations in the drug levels due to the loss of the drug into the liver and kidneys.

The maximum phenothiazine level was 137.8 μg./ml. of blood serum/100 lb. of live body weight and the maximum phenothiazone level was 342.0 μg./ml. of blood serum/100 lb. of live body weight; both at 24 hours post-treatment. No trace of drug was detected in the blood at 120 hours.

Bile.—Chronological levels of phenothiazine and phenothiazone are shown (fig. 3). Phenothiazine and phenothiazone were recovered in the bile at one hour post-treatment. The one-hour concentration of phenothiazine was approximately twice that of phenothiazone. At three hours, the concentration of phenothiazone increased nearly sevenfold over the preceding hour, and its concentration was twice that of phenothiazine.

The levels of the drug and its derivatives in bile were irregular; the levels showed sudden increases and decreases. At 14 and 16 hours, phenothiazine was higher in concentration than phenothiazone.

Maximum drug levels in the bile were detected at 16 hours post-treatment. The levels were 72.9 mg. of phenothiazine and 64.8 mg. of phenothiazone. No trace of drug in bile was detected at 120 hours.

Urine.—The drug levels in the urine were recorded to correlate the levels of the drug eliminated with the levels of the drug in other parts of the animal body and not primarily as to amount of drug eliminated. Chronological levels of phenothiazine and phenothiazone in urine are shown (fig. 4).

The first appearance of phenothiazone

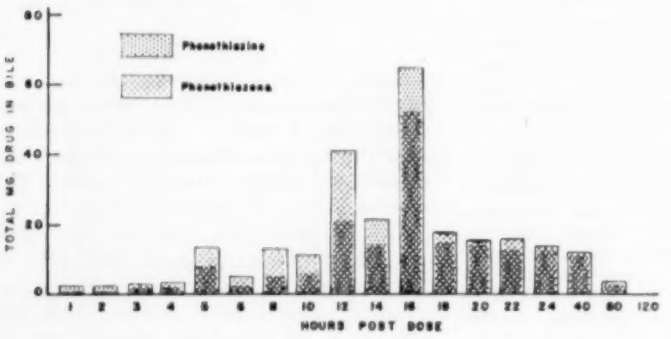


Fig. 3—Bile chronological levels of phenothiazine and phenothiazone in dairy calves.

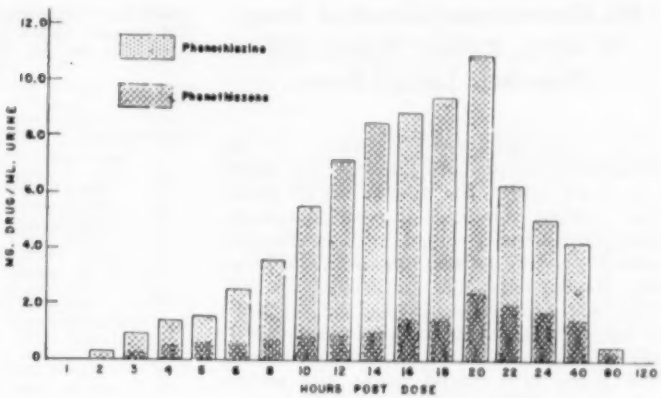


Fig. 4—Chronological elimination of phenothiazine and phenothiazone in urine from dairy calves.

in the urine occurred at two hours post-treatment and phenothiazine appeared one hour later. Maximum drug levels occurred at 20 hours. The maximum level of phenothiazone was 10.8 mg./ml. of urine and maximum level of phenothiazine was 2.40 mg./ml. of urine. No trace of drug was detected in urine at 120 hours.

The major constituent of the drug in urine was the urinary conjugate of leukophenothiazone. The bovine urinary conjugate has been isolated and identified in this laboratory.

Discussion

Until recently, it has been widely held that absorption from the ruminant stomach does not occur.[4] The present work may indicate that further revision of the concept of rumen absorption may be necessary. Because of the insolubility of phenothiazine in water, the particle size may have much to do with its absorption through the rumen walls. Water-soluble phenothiazone was more easily absorbed into the blood in these trials.

The great variations in the bile volume of the gallbladder in these trials were not due to apparent physical disorders. Bile is secreted continuously by the hepatic cells but the rate at which the gallbladder empties varies with food consumed and other factors. The differences of the bile volume may cause some discrepancy in the drug levels calculated on a per milliliter basis.

The first appearance of the drug in the urine occurred within two hours post-treatment. At that time, the drug appeared only as phenothiazone. Phenothiazine was not detectable until the third hour.

Summary

1) Concentrations of phenothiazine and its derivatives in the blood serum of 18 calves involved in a chronological study fluctuated sharply with several peak levels. On the basis of micrograms of drug per milliliter of blood serum only, the maximum levels were 61.0 μg. of phenothiazine and 115.0 μg. of phenothiazone at 14 hours post-treatment. When the drug levels were based on a 100-lb. live body weight, the maximum levels (137.8 μg. of phenothiazine and 342.0 μg. of phenothiazone) did not occur until 24 hours.

2) Maximum drug levels in bile (72.9 mg. of phenothiazine and 64.8 mg. of phenothiazone) were detected at 16 hours.

3) Maximum drug levels in the urine (2.40 mg. of phenothiazine/ml. and 10.80 mg. of phenothiazone/ml.) were detected at 20 hours.

4) No traces of drug were detected in blood serum, bile, or urine at 120 hours post-treatment.

References

[1] Clare, N. T.: A Photosensitized Keratitis in Young Cattle Following the Use of Phenothiazine as an Anthelmintic. II. The Metabolism of Phenothiazine in Ruminants. Austral. Vet. J., *23*, (1947): 340–344.

[2] Harpur, R. P., Swales, W. E., and Denstedt, O. F.: The Fate of Phenothiazine in the Gastrointestinal Tract of the Sheep. Canad. J. Res., *28*, (1950): 143–161.

[3] Swales, W. E., and Collier, H. B.: Studies on Effects and Excretion of Phenothiazine when Used as an Anthelmintic for Sheep. Canad. J. Res., *18*, (1940): 279–287.

[4] Dukes, H. H.: The Physiology of Domestic Animals. 7th ed. Comstock Publishing Associates, Ithaca, N. Y., 1955.

III. Chronological Levels of Drug in Liver, Kidney, Spleen, and Mesenteric Lymph Nodes

In conjunction with the studies of the metabolism of phenothiazine in the digestive tract and blood serum of 18 grade dairy calves, it was of interest to investigate the organs involved in drug detoxification and filtration. Enlarged kidneys and spleen, nephritis, and hemolytic anemia have been reported as toxic reactions to phenothiazine. A study of the levels of phenothiazine in the liver, kidney, spleen, and mesenteric lymph nodes was undertaken to establish a future basis for interpretation of toxic response to phenothiazine by these tissues.

MATERIALS AND METHODS

Eighteen grade calves ranging in weight from 150 to 525 lb. were used in this study. The calves were slaughtered at designated periods after administration of 101.3 Gm. of drench-grade phenothiazine (100.0 Gm. of phenothiazine, N.F.). The liver, spleen, and kidneys were removed, weighed, and aliquots were removed for analysis. The mesenteric lymph nodes were removed and a sample selected for analysis.

The tissues were cut into small cubes (2.5 by 2.5 cm.) to facilitate a representative sampling of the organ and to expose surfaces for thorough extraction. Two 20.0-Gm. samples each of liver, spleen, kidney (half from each kidney), and lymph nodes were weighed into 250.0-ml. beakers. To one sample of each tissue was added 50.0 ml. of distilled water and, to the other, 50.0 ml. of 95 per cent ethanol. The beakers were covered to prevent loss of solvent by evaporation and were placed in a hot-air oven at 80 C. for four hours. The samples were left at room temperature for an additional 24 hours for maximum extraction. After filtration, each filtrate sample was made up to 50.0 ml. in volume with its respective solvent. The samples were stored in the refrigerator until cool and were refiltered if necessary to obtain utmost clarity.

Analysis of phenothiazine and phenothiazone proceeded according to methods outlined in other parts of this series.

RESULTS AND DISCUSSION

Chronological levels of the drug in the liver, spleen, and both kidneys are shown (table 1).

Liver.—First appearance of phenothiazone in the liver was at two hours post-treatment. Phenothiazine was first detected at three hours. Maximum weight of phenothiazine in a liver was 0.2574 Gm. at 24 hours; maximum weight of phenothiazone was 0.1333 Gm. at 16 hours. No trace of drug could be detected at 120 hours.

Kidney.—In the kidney, phenothiazine was first detected at two hours and phenothiazone at three hours post-treatment. Kidney maximum drug weights were 0.0413 Gm. of phenothiazine at 16 hours and 0.0394 Gm. of phenothiazone at 24 hours. No drug was detected at 120 hours.

Spleen.—Phenothiazone was detected in the spleen at one hour post-treatment and

TABLE 1—Chronological Levels of Drug in Liver, Kidney, and Spleen from Dairy Calves Administered 100 Gm. of Phenothiazine, N.F. (Drench Grade)

Hours post-treatment	Liver		Kidney		Spleen	
	P-zine*	P-zone†	P-zine	P-zone	P-zine	P-zone
1	0	0	0	0	0	0.0009
2	0	0.0392	0.0025	0	0	0.019
3	0.0523	0.0239	0.0038	0.005	0.0054	0.0059
4	0.0271	0.0396	0.0015	0.0064	0.0056	0.0029
5	0.0357	0.0027	0.0029	0.0075	0.0226	0.0486
6	0.0591	0.0237	0.0051	0.007	0.009	0.03
8	0.071	0.0041	0.003	0.0113	0.0029	0.0023
10	0.1037	0.0083	0.0037	0.0176	0.0088	0.0126
12	0.0821	0.0023	0.0118	0.0281	0.0046	0.0003
14	0.1346	0.0075	0.0057	0.0185	0.0072	0.0006
16	0.0409	0.1333	0.0413	0.0105	0.0114	0.0254
18	0.1005	0.008	0.0108	0.0198	0.0043	0.0014
20	0.2142	0.0121	0.012	0.0258	0.0111	0.0007
22	0.1368	0.0137	0.0256	0.0109	0.0091	0.0036
24	0.2574	0.0107	0.012	0.0394	0.0223	0.0042
40	0.1583	0.009	0.0055	0.0279	0.0081	0.0012
80	0.0308	0.0062	0.0066	0.0252	0.0036	0.0024
120	0	0	0	0	0	0

* Unchanged phenothiazine (total grams of drug).
† Phenothiazone and other water-soluble, oxygenated phenothiazine derivatives (total grams of drug).

TABLE 2—Chronological Levels of Drug in Mesenteric Lymph Nodes from Dairy Calves Administered 100 Gm. of Phenothiazine, N.F. (Drench Grade)

Hours post-treatment	Phenothiazine, μg./Gm. fresh tissue	Phenothiazone,* μg./Gm. fresh tissue
1	0	0
2	0	0
3	0	0
4	0	0
5	0	0
6	0	0
8	15.6	0
10	25.0	0.6
12	28.1	8.1
14	59.4	6.3
16	31.3	4.4
18	25.0	2.5
20	20.0	2.5
22	15.0	3.8
24	12.5	3.8
40	9.4	2.5
80	1.3	2.5
120	0	0

* Phenothiazone and other water-soluble, oxygenated phenothiazine derivatives.

phenothiazine at three hours. Maximum weight of drug in a spleen was 0.0226 Gm. of phenothiazine and 0.0486 Gm. of phenothiazone at five hours. No drug was detected at 120 hours.

Mesenteric Lymph Nodes.—Chronological levels of the drug are shown (table 2). Phenothiazine first appeared at eight hours and phenothiazone at ten hours post-treatment. The maximum drug levels for mesenteric lymph nodes were 59.4 μg. of phenothiazine/Gm. of fresh tissue at 14 hours and 8.1 μg. of phenothiazone/Gm. of fresh tissue at 12 hours. No drug was detected at 120 hours.

Comparison of the chronology of first drug appearance and maximum drug levels in the liver and kidneys showed a contrasting situation. At two hours post-treatment, phenothiazone first appeared in the liver and phenothiazine in the kidneys. Maximum drug concentrations occurred at 16 hours for phenothiazone in liver and phenothiazine in the kidneys. The reverse occurred at 24 hours, with a maximum concentration of phenothiazine in the liver and phenothiazone in the kidneys.

The first drug appearance in the liver, kidneys, and spleen occurred six hours prior to the first drug appearance in the mesenteric lymph nodes. Reasons for the delay of the appearance of phenothiazine and its derivatives in the mesenteric lymph nodes were not fully understood.

SUMMARY

1) The first appearance of drug in the liver was phenothiazone at two hours post-treatment. Phenothiazine was detected at three hours. Maximum drug levels were at 16 hours for phenothiazone and 24 hours for phenothiazine.

2) Phenothiazine was first detected in the kidneys at two hours and phenothiazone at three hours post-treatment, with maximum drug levels at 16 hours for phenothiazine and 24 hours for phenothiazone. The chronology of drug appearance in the kidneys was the reverse of the situation existing in the liver.

3) In the spleen, the first appearance of drug was phenothiazone at one hour and phenothiazine at three hours post-treatment. Maximum drug levels occurred at five hours for phenothiazine and phenothiazone.

4) In the mesenteric lymph nodes, the appearance of drug was delayed. Phenothiazine appeared at eight hours and phenothiazone at ten hours post-treatment. Maximum drug levels occurred at 12 hours for phenothiazone and 14 hours for phenothiazine.

5) No drug was detected in liver, kidneys, spleen, or mesenteric lymph nodes at 120 hours post-treatment.

References

[1] Collier, H. B., and Allen, D. E.: The Hemolytic Action of Phenothiazine Derivatives. Canad. J. Res., *20*, (1942): 283–290.

[2] Woodhouse, C. A., Reid, J. J., and Schmidt, H.: Fifty-Fourth Annual Report Texas Agric. Exper. Sta. (1942): 111–112.

IV. Chronological Total Drug Recovery

The present investigation in a series on the metabolism of phenothiazine and its derivatives was undertaken to determine the chronological total recovery of non-eliminated drug from dairy calves dosed with 100.0 Gm. of phenothiazine, N.F. Chronological total elimination of the drug in feces and urine or chronological total drug levels in the blood of the calves used in these trials were not measured.

The duration of appearance of phenothiazine and its derivatives within the digestive tract and in the various organs may be related to toxic response in certain animals.

Metabolism of phenothiazine had been studied by DeEds *et al.*[1] in rats, rabbits, and man, and by Swales and Collier [2] and Gordon and Lipson [3] in sheep. Literature on phenothiazine therapy contains many references to toxic responses by treated animals. Enlarged kidneys and hemolytic anemia have been reported in horses which manifested a toxic response.[4–6] A marked hemolytic anemia has been reported in other mammals following large doses of phenothiazine. Woodhouse *et al.*[7] reported an anemia in cattle, and McNally [8] reported an anemia in sheep. The relationship of phenothiazine therapy to such anemia has not been established. Severe anemias with fatal results have been described from doses as low as 15.0 Gm. per horse by Mitrovic.[9]

Reactions to phenothiazine by cattle included constipation, weakness in hind quarters, lowered temperature, photosensitization keratitis, prostration, coma, and

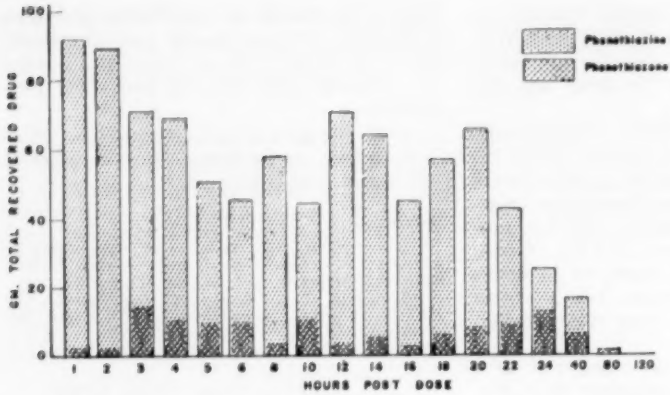


Fig. 1—Total amount of recovered phenothiazine and phenothiazone in dairy calves.

death. These reactions have been recorded by Clare *et al.*,[10,11] Duthby and Lewis,[12] and Whitten *et al.*[13]

MATERIALS AND METHODS

Eighteen dairy calves were each dosed orally with 101.3 Gm. of drench-grade phenothiazine (100.0 Gm. phenothiazine, N.F.). The calves were slaughtered at selected hours post-treatment and the digestive tract contents, liver, spleen, kidneys, and bile were analyzed for total weight of phenothiazine and phenothiazone.

RESULTS AND DISCUSSION

The total amounts of phenothiazine and phenothiazone recovered from the calves in these trials are shown (fig. 1).

In the first hour post-treatment, 91.48 Gm. of phenothiazine and 2.75 Gm. of phenothiazone were recovered. No trace of phenothiazine was detected in liver, spleen, or kidneys in the first hour.

A peak level of 14.08 Gm. of phenothiazone was recovered at three hours. The levels of phenothiazine and phenothiazone fluctuated sharply between designated periods.

At 80 hours post-treatment, 0.04 Gm. of phenothiazine and 0.97 Gm. of phenothiazone remained in the animal. No traces of drug were detected at 120 hours.

Noneliminated phenothiazine and its derivatives recovered from the digestive tract contents and organs of the test animals at one hour post-treatment totaled 94.2 Gm. of the 100.0-Gm. dose of phenothiazine, N.F. Phenothiazine and phenothiazone were detected in blood at one hour; drug levels were not determined on a total blood volume basis. A considerable part of the unrecovered drug evidently was present in the circulatory system. Total concentrations of drug eliminated in the feces and urine were not available.

The purpose of this study was to record only total amounts of drug available to affect the tissues and not to record which tissues are affected by phenothiazine and its derivatives. At 40 hours post-treatment, only 22.1 Gm. of phenothiazine and derivatives were distributed in small amounts throughout the digestive tract, liver, kidneys, spleen, mesenteric lymph nodes, and bile.

No traces of drug were detected in the experimental animals at 120 hours post-treatment.

SUMMARY

Eighteen grade dairy calves of mixed breeds and of both sexes were each dosed orally with 101.3 Gm. of drench-grade phenothiazine (100.0 Gm. of phenothiazine, N.F.). The calves were slaughtered at designated periods.

The post-treatment periods used and the total concentrations of phenothiazine and phenothiazone, respectively, were: 1 hour, 91.5 Gm., 2.7 Gm.; 2 hours, 89.1 Gm., 2.0 Gm.; 3 hours, 70.6 Gm., 14.1 Gm.; 4 hours, 69.0 Gm., 10.6 Gm.; 5 hours, 50.8 Gm., 9.7 Gm.; 6 hours, 45.4 Gm., 10.0 Gm.; 8 hours, 57.9 Gm., 3.6 Gm.; 10 hours, 44.3 Gm., 11.3 Gm.; 12 hours, 70.4 Gm., 3.8 Gm.; 14 hours, 63.8 Gm., 5.6 Gm.; 16 hours, 44.5 Gm., 2.4 Gm.; 18 hours, 56.4 Gm., 5.9 Gm.; 20 hours, 65.4 Gm., 8.2 Gm.; 22 hours, 42.6 Gm., 8.9 Gm.; 24 hours, 25.4 Gm., 12.9 Gm.; 40 hours, 16.2 Gm., 5.9 Gm.; and 80 hours, 0.04 Gm., 0.97 Gm.

No drug was detected at 120 hours post-treatment.

References

[1] DeEds, F., Eddy, C. W., and Thomas, J. O.: Studies on Phenothiazine. V. Fate of Phenothiazine in the Body. J. Pharmacol. and Exptl. Therap., *64*, (1938): 250–254.

[2] Swales, W. E., and Collier, H. B.: Studies on Effects and Excretion of Phenothiazine When Used as an Anthelmintic for Sheep. Canad. J. Res., *18*, (1940): 279–287.

[3] Gordon, H. McL., and Lipson, M.: Preliminary Examination of the Anthelmintic Efficiency of Certain Compounds Related to Phenothiazine. J. Council Scient. and Indust. Res. Austral., *13*, (1940): 173–177.

[4] Collier, H. B., and Allen, D. E.: The Hemolytic Action of Phenothiazine Derivatives. Canad. J. Res., *20*, (1942): 283–290.

[5] Hatcher, W. L.: Phenothiazine Poisoning in Horses. North Am. Vet., *22*, (1941): 159–160.

[6] Herrod-Taylor, E. E.: Phenothiazine Overdosage or Idiosyncrasy in the Equine. Vet. Rec., *60*, (1948): 71.

[7] Woodhouse, C. A., Reid, J. J., and Schmidt, H.: Fifty-Fourth Annual Report, Texas Agric. Exper. Sta. (1942): 111–112.

[8] McNally, W. D.: The Effect of Varying Doses of a Commercial Phenothiazine upon Lambs. J.A.V.M.A., *102*, (1943): 170–175.

[9] Mitrovic, M.: Two Cases of Fatal Poisoning in Horses Treated with Therapeutic Doses of Phenothiazine. Zootech. Vet. Milan, *3*, (1948): 157–160.

[10] Clare, N. T.: A Photosensitized Keratitis in Young Cattle Following the Use of Phenothiazine as an Anthelmintic. II. The Metabolism of Phenothiazine in Ruminants. Austral. Vet. J., *23*, (1947): 340–344.

[11] Clare, N. T., Whitten, L. K., and Filmer, D. B.: A Photosensitized Keratitis in Young Cattle Following the Use of Phenothiazine as an Anthelmintic. III. Identification of the Photosensitizing Agent. Austral. Vet. J., *23*, (1947): 344–348.

[12] Duthby, B. L., and Lewis, R. W. E.: Eye Trouble Attributable to Photosensitization Following the Use of Phenothiazine in Bovines. East Afric. Agric. J., *14*, (1949): 155–156.

[13] Whitten, L. K., Clare, N. T., and Filmer, D. B.: A Photosensitized Keratitis in Cattle Dosed with Phenothiazine. Nature, *157*, (1946): 232.

Infectious Bovine Rhinotracheitis

J. H. GILLESPIE, V.M.D.; K. M. LEE, M.D., Ph.D.; J. A. BAKER, D.V.M., Ph.D.

Ithaca, New York

A NEWLY recognized clinical syndrome characterized by respiratory signs was reported in cattle from California in 1954 by McIntyre,[1] by McKercher *et al.*,[2] and by Schroeder *et al.*[3] McIntyre described successful transmission of the disease by inoculation of a mixture of blood, nasal discharges, and feces from sick calves and also observed spread by pen contact.

In 1955, a similar illness was found to occur in cattle kept in Colorado feedlots by Jensen *et al.*[4] and also by Miller.[5] Chow *et al.*[6] and McKercher *et al.*[7] showed that the infectious agent from Colorado was similar to the one from California. Further studies on the agent permitted both Chow's and McKercher's groups to conclude that this etiological agent was a virus.

Recently, Madin *et al.*[8] cultivated the virus in tissue culture, using bovine kidney cells; it grew well and caused cytopathogenic effects. These effects were reproducible in serial passages and bacteria-free fluids from infected tissues reproduced the characteristic disease in calves. These studies offered further proof of viral causation.

In the excellent reports on this disease, now designated infectious bovine rhinotracheitis, the workers emphasized its importance in cattle of the western United States. Our investigation was designed to study possible occurrence and effects of infectious bovine rhinotracheitis virus on cattle in the eastern United States.

MATERIALS AND METHODS

Cultivation in Tissue Culture.—For tissue culture preparation, bovine fetuses between 2 to 7 months of age furnished kidney cortex as the source of cells which were prepared after the method of Dulbecco and Vogt.[9] The nutrient fluid used was Earle's balanced salt solution (80%),[10] to which had been added lactalbumin hydrolysate (10%), horse serum (10%), penicillin (100 units/ml.), streptomycin (100 μg./ml.), and mycostatin (100 units/ml.). After six or seven days, the initial nutrient fluid was removed and replaced by 2.0 ml. of maintenance fluid that differed only in its amount of horse serum (1%) and Earle's medium (89%). On occasions, 25 μg./ml. of erythromycin was added to inhibit the growth of certain bacterial organisms present in the nasal passages of some calves.

Work began with a strain of virus which was obtained originally from an animal with a clinical infection (in Colorado) and then transferred for five serial passages in tissue culture. Upon receipt, this tissue-cultured virus * was inoculated intratracheally into a calf. Two days after its temperature became elevated, this calf was killed for necropsy. Pneumonic areas from the lung were removed, distributed in vials, and placed under dry-ice refrigeration. This material was used to infect other calves and to initiate a tissue culture series.

Lung tissue was thawed, weighed and, with a glass grinder, made into a 10 per cent suspension in maintenance fluid. Coarse particles were sedimented by centrifugation and, from the supernatant, tenfold dilutions were prepared in maintenance fluid. From each dilution, 0.2 ml. was inoculated into each of five tissue culture tubes prepared as above. The tubes then were placed in a roller drum for 12 days and examined daily for cell degeneration. A tube in which the cells were degenerated was considered positive for virus, while normal cellular appearance was counted as negative. Virus content was expressed as an end point calculated by the Reed-Muench formula.[11] In subsequent transfers, tissue culture fluid from infected tubes was removed and transferred to other tubes for 100 serial passages. At various passage intervals, virus content was determined as described for lung tissue, except the number of tubes used for each dilution was increased to ten or more. Lung from the calf originally infected showed a virus end point of $10^{-4.9}$ per 0.2 ml. of inoculum. The 2, 8, 17, 27, 34, 49, 58, 67, and 100 tissue culture transfers had virus end points that ranged from $10^{-5.8}$ to $10^{-6.4}$ per 0.2 ml. This method was used in all the tests that follow.

Calf Inoculations.—With one exception, dairy calves 4 to 8 months of age were used in these studies. Of the 19 calves, 15 were taken from a disease-free herd maintained by this Institute under special conditions, while 4 were purchased from a farm in the vicinity of Ithaca, N. Y.

From the Veterinary Virus Research Institute, New York State Veterinary College at Cornell University, Ithaca, N. Y.

These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and Cornell University, NR-130-352, Ithaca, N.Y.

Dr. Lee is now veterinary consultant, United Nations Command, Office of the Economic Coordinator for Korea.

* Provided through the courtesy of Dr. Charles J. York, Research Department, Pitman-Moore Co., Indianapolis, Ind.

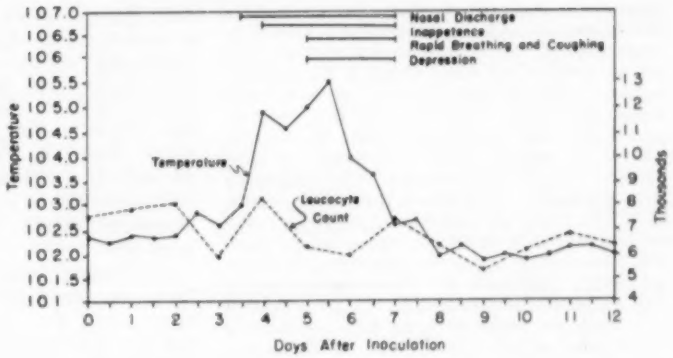


Fig. 1—Clinical features of infectious rhinotracheitis in a calf inoculated intranasally.

When placed on test, all were kept in isolation units. Each calf was allowed to adjust to its new environment for one week prior to inoculation. During this period, temperatures were taken daily and each animal was observed closely for any signs of illness. All appeared normal.

Four calves were inoculated intranasally and 3 intratracheally, each with 20.0 ml. of a 5 per cent suspension of lung from the infected calf mentioned above; 4 were inoculated intranasally and 4 intratracheally, each with 20.0 ml. of fluids from the eighth tissue culture transfer diluted 1:20. One calf was exposed to an infected calf. After the exposed calf showed signs of illness, it was removed and placed in a unit with another susceptible calf. This procedure was repeated two additional times, thus making four serial exposures. After inoculation or exposure, calves were examined daily for signs of illness. Temperatures were taken twice each day. All calves were bled for serological tests before inoculation and then after 12 days or longer if still on test. Some calves were killed for necropsy during the active stage of infection and their tissues were saved for further study.

Presence and Persistence of Virus in Calves.—Daily nasal swabs were made for five days after inoculation on 2 calves given virus intranasally and on 2 infected intratracheally. In securing nasal exudate, a cotton swab was introduced into each nasal passage, twirled, and the sodden swab was replaced in 2.0 ml. of phosphate-glutamate-albumin medium [12] before freezing. Nasal swabs also were procured from 6 recovered calves, respectively, on postinoculation days 22, 26, and from 40 to 60. Additional studies were made on 5 calves which had been inoculated intranasally with tissue-cultured or cattle origin virus, and on 5 calves inoculated intratracheally. On the day that the body temperature became elevated and on each succeeding day thereafter, 1 calf inoculated by each route was killed and various tissues, including lung, blood, mediastinal lymph node, tracheal mucous membrane, kidney, liver, and spleen were removed, quick frozen, and placed under dry-ice refrigeration. Also, nasal exudate was obtained from each calf at the time of necropsy. All materials were tested by the tissue culture method for the presence and concentration of virus.

Immunity Tests.—The immunity of 8 calves which had shown signs of illness following inoculation intratracheally, intranasally, or by contact exposure was challenged by inoculation 12 to 90 days later. Each was given intranasally 20.0 ml. of a 1:20 dilution of cattle lung suspension or eighth tissue culture transfer. Serums from these calves

TABLE 1—Concentration of Infectious Bovine Rhinotracheitis Virus in Tissues from Calves Killed for Necropsy

Tissue examined	Days * following intranasal inoculation					Days * following intratracheal inoculation				
	1	2	3	4	5	1	2	3	4	5
Nasal exudate	+(5.1)†	+(6.4)	+(5.8)	+(5.8)	+(4.8)	—	—	—	+(1.7)	+(5.2)
Trachea	+(1.5)	+(1.8)	+(1.4)	—	+(2.3)	NT‡	NT	NT	+(1.5)	+(0.6)
Lung	+(2.8)	—	—	—	+(3.2)	+(4.1)	+(4.9)	+(4.2)	+(4.8)	+(1.8)
Mediastinal lymph node	—	—	—	—	+(1.6)	NT	+(2.5)	+(1.8)	+(2.2)	+(0.8)
Blood	—	—	—	—	—	—	—	—	+(0.8)	+(0.3)
Spleen	—	—	—	—	—	—	—	—	—	—
Kidney	—	—	—	—	—	—	—	—	—	—
Liver	—	—	—	—	—	—	—	—	—	—

* Tests began day temperature became elevated.

† Minus sign means no virus found in 10^{-1} dilution or in undiluted blood and plus sign means virus present; titers expressed as log t.c.i.d.$_{50}$ per 0.2 ml. of inoculum and calculated by method of Reed and Muench.

‡ NT = not tested.

taken before and after inoculation were tested for neutralizing antibodies against virus cultivated in tissue culture. All serums were heated at 56 C. for 30 minutes and then diluted 1:2.5 in maintenance fluid. Tenfold dilutions of virus that ranged from 1:500 to 1:5,000,000 were then prepared with cold maintenance fluid. In the test, 1.0 ml. of diluted serum was added in equal amount to each virus dilution. The components were thoroughly mixed, placed under refrigeration at 6 C. for two hours, and then 0.2 ml. of each mixture was added to each of five tissue culture tubes. The tissue culture tubes were kept for 12 days and periodically examined for cytopathogenic effects. The serum-neutralization index of the convalescent serum was computed as the difference between the $t.c.i.d._{50}$ of virus mixed with preinoculation serum and the $t.c.i.d._{50}$ of the virus mixed with postinoculation serum.

RESULTS

All calves inoculated intranasally or intratracheally showed signs of illness. By contact exposure, the disease was transmitted in series for three passages but not on the fourth passage.

Calves, whether inoculated with infected calf lung or tissue-cultured virus, showed signs of illness 18 to 36 hours after intratracheal inoculation, 72 to 120 hours after intranasal inoculation, and 96 to 144 hours after contact exposure. Typical clinical features in a calf are shown (fig. 1). All infected calves had a monophasic temperature reaction for two to six days and all showed respiratory signs except 1 exposed calf. Shortly after the rise in temperature, a serous nasal discharge developed that occasionally became mucopurulent. The respiratory rate increased and breathing became shallow. Most calves coughed and showed salivation; some showed inappetence, depression, and lacrimation; and 1 developed diarrhea. The leukocyte count remained within normal range. All calves recovered except those killed for special studies.

Studies on presence and persistence of virus in inoculated calves showed that, after virus was placed in the nasal passages, it was recoverable the following day and increased in amount on each succeeding day, reaching maximum concentration by the fifth day after inoculation (fig. 2). In contrast, virus given intratracheally was not present in nasal passages until the fifth day after inoculation but then increased rapidly. Virus was not found in nasal exudate, in calves after recovery. Virus reached its highest concentration in nasal exudate of any tissue tested following intranasal inoculation (table 1). Of 5 calves inoculated intranasally, virus was found in the trachea of 4, in the lungs of 2, and in the mediastinal lymph nodes of 1. Of 5 calves inoculated intratracheally, virus was found in the lungs of 5, trachea of 2, mediastinal lymph nodes of 4, the blood of 2, and the nasal passages of 2.

The results of the serum-neutralization

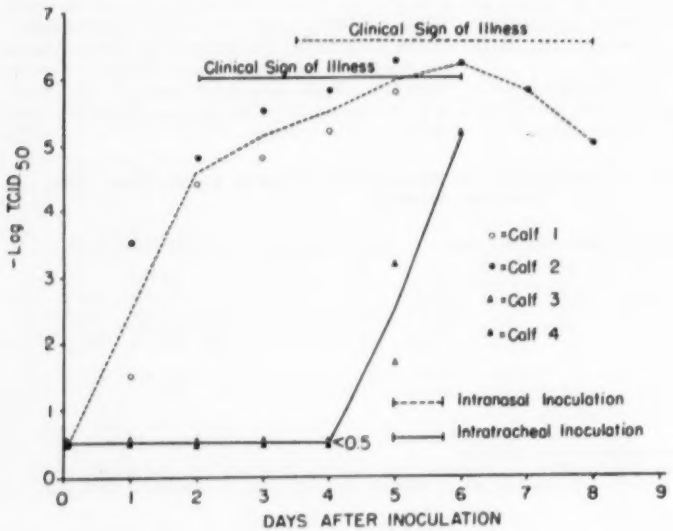


Fig. 2—Concentration of virus in nasal exudate at daily intervals after inoculation.

tests and immunity tests are shown (table 2). The convalescent serums of all calves whose serum was tested more than three weeks after inoculation neutralized the virus with indexes that ranged from 50 to 10,000. One calf tested 12 days afterward showed neutralizing antibodies, whereas another did not. No definite signs of illness developed in these recovered calves, although 5 showed a slight increase in temperature, for one reading only, four to five days after inoculation. Since this virus caused typical illness in susceptible calves, it was concluded that calves were immune after recovery.

TABLE 2—Immunity to Infectious Bovine Rhinotracheitis

Calf No.	Method of infection	Serological findings *		
		Days after exposure or inoculation	Neutralization index in tissue culture	Response to test virus
412	Intratracheal	12	0	Immune
414	Contact	22	50	Immune
415	Intranasal	90	10,000	Immune
417	Intratracheal	26	2,000	Immune
422	Intratracheal	12	58	Immune
423	Contact	41	600	Immune
424	Contact	55	1,600	Immune
425	Intranasal	31	500	Immune

* The neutralization index is computed as the difference between the t.c.i.d.$_{50}$ of virus and preinoculation serum and the t.c.i.d.$_{50}$ of virus and postinoculation serum.

INCIDENCE OF NEUTRALIZING ANTIBODIES IN CATTLE IN THE EASTERN UNITED STATES

From each of 43 dairy herds being tested for brucellosis, five blood samples † were selected at random, pooled, and tested for neutralizing antibodies. In addition, tests were made of preinoculation and postinoculation serums that had been collected in 1941 [13] from 2 calves inoculated with calf pneumonia and of another calf from a dairy in New Jersey, also collected in 1941. Serums from cattle convalescent from so-called shipping fever were tested. Serums from 5 calves that had been infected with virus diarrhea, 5 with *Leptospira pomona*, and 5 with *Miyagawanella bovis* were also tested. Serums from susceptible calves before inoculation and serums with neutralization indexes above 3,150 for infectious bovine rhinotracheitis were included in each test as controls. Serums diluted 1:5 and 1:50 were tested as described above.

All tubes containing the final serum dilution of 1:100 showed a cytopathogenic effect and were discarded. Among the serums diluted 1:10, those from susceptible calves; from shipping fever-convalescent calves; from animals immune to either *L. pomona*, virus diarrhea, or *M. bovis;* the preinoculation and postinoculation serums from the 2 calves infected with a virus of calf pneumonia in 1941; and serums from the pools of 38 New York State cattle herds did not neutralize the virus. The serum pools from five New York State cattle herds, the serum from the New Jersey dairy calf, and serum from infectious bovine rhinotracheitis-immune calves neutralized the virus.

† Kindly supplied by Dr. T. F. Benson, director, Diagnostic Laboratory, Department of Pathology and Bacteriology, New York State Veterinary College, Cornell University, Ithaca, N. Y.

COMPARISON OF INFECTIOUS BOVINE RHINOTRACHEITIS VIRUS WITH OTHER AGENTS WHICH PRODUCE DISEASE IN CATTLE

Whenever possible, cross-immunity tests that were reciprocal were made between infectious bovine rhinotracheitis virus, virus diarrhea virus, *L. pomona*, and *M. bovis*. In the tests, calves that had been infected with a particular agent were inoculated with the same agent and, in sequence, with all the others at intervals of 14 days (table 3). Methods of producing infection are reported in previous publications,[14-18] except that used for infectious bovine rhinotracheitis which is reported here.

Results showed that infectious bovine rhinotracheitis virus immunized against itself, but calves protected against virus diarrhea virus, *L. pomona*, or *M. bovis*

TABLE 3—Cross-Immunity Tests in Calves Between Infectious Bovine Rhinotracheitis Virus and Other Agents Which Produce Infection in Cattle

Immunizing agent	Test agent *			
	Virus diarrhea virus	Rhinotracheitis agent	*Leptospira pomona*	*Miyagawanella bovis*
Virus diarrhea virus	0/12	12/12	10/10	5/5
Rhinotracheitis virus	4/4	0/9	Not tested	Not tested
M. bovis	5/5	5/5	5/5	0/5†
L. pomona	5/5	2/2	0/10	5/5

* Denominator indicates number of animals used; numerator indicates number showing signs of illness.

† *Miyagawanella bovis* did not produce illness and the virus persisted; therefore, the result is equivocal.

were not immune to infectious bovine rhinotracheitis. Also, calves immune to infectious bovine rhinotracheitis were not immune to virus diarrhea virus.

DISCUSSION

Tissue culture methods applied in a study of the transmissible respiratory disease in cattle, designated infectious bovine rhinotracheitis, yielded a cytopathogenic agent which reproduced the disease in susceptible calves. Studies of this agent led to the conclusion that it was a virus.[8] The studies reported herein are in agreement with these findings.

Titration of various tissues during the course of illness has shown that, following intranasal inoculation, there was rapid proliferation of virus in high concentration in the nasal mucosa only, and other tissues contained little or no virus. This finding correlated with clinical signs of illness and clearly shows that the disease is aptly named infectious bovine rhinotracheitis.

A neutralization test was developed for this virus and shown to be specific, in so far as no cross reactions were found with other infectious agents for cattle. Using this test, it was shown that the virus was neutralized by serums from calves which had recovered clinically from infection. Also, 12 per cent of pooled serums from 38 cattle herds in New York neutralized virus, as did a calf serum which had been collected in 1941 at Princeton, N. J. These findings strongly suggest that this disease has been occurring in cattle in the eastern United States for a long time and is not an infrequent infection.

Virus did not persist in recovered animals. It did spread readily by contact exposure but, other than continued maintenance by recently infected animals, no epizootiological explanation has been found.

Calves, after recovery from initial infection, were immune for at least three months to subsequent exposure. This suggests the possible value of vaccination, especially in feedlots where serious economic losses have been reported. Certainly, extensive efforts should be made to establish attenuated strains of virus for vaccine purposes. This may be possible by tissue culture methods.

SUMMARY

Infectious bovine rhinotracheitis virus has been maintained by tissue culture in bovine kidney cells for 100 serial transfers. It produced cytopathogenic effects. Temperature elevations that lasted from two to six days were induced within 18 to 36 hours when virus was given intratracheally, and within 72 to 120 hours when given intranasally. The virus was transferred from infected calves to others by contact. In addition to increased temperature, infected calves showed salivation, lacrimation, inappetence, and depression. The calves developed neutralizing antibodies and, after recovery, were immune to subsequent inoculation of virus.

In calves inoculated intranasally, the virus concentration was greatest in nasal exudate. Calves inoculated intratracheally showed a high concentration initially in the lungs and later in nasal mucosa. After intranasal inoculation, virus appeared in nasal mucus the following day in low concentrations and gradually increased, reaching its highest titer five days after inoculation. Virus was not recovered in nasal exudate 22 to 60 days after inoculation.

Neutralizing antibodies were found in some cattle from dairy herds in New York and also in a calf serum collected in 1941 in New Jersey. Therefore, it was considered probable that infectious bovine rhinotracheitis occurs in cattle in the eastern United States and has been present for a long time.

References

[1] McIntyre, R. W.: Experimental Studies of Acute Upper Respiratory Infection in Cattle. J.A.V.M.A., *125*, (1954): 473.

[2] McKercher, D. G., Moulton, J. E., and Jasper, D. E.: Virus and Virus-like Disease Entities New to California. Proc. U. S. Livestock San. A. (1954): 260–269.

[3] Schroeder, R. J., and Moys, M. D.: An Upper Respiratory Infection of Dairy Cattle. J.A.V.M.A., *125*, (1954): 471.

[4] Jensen, Rue, Griner, L. A., Chow, T. L., and Brown, W. W.: Infectious Rhinotracheitis in Feedlot Cattle. I. Pathology and Symptoms. Proc. U. S. Livestock San. A. (1955): 189–199.

[5] Miller, N. J.: Infectious Necrotic Rhinotracheitis of Cattle. J.A.V.M.A., *126*, (1955): 463.

[6] Chow, T. L., Deem, A. W., and Jensen, Rue: Infectious Rhinotracheitis in Cattle. II. Experimental Reproduction. Proc. U. S. Livestock San. A. (1955): 168–172.

[7] McKercher, D. G., Moulton, J. E., and Kendrick, J. W.: Recent Developments on Upper Respiratory Disease of Cattle. Proc. U. S. Livestock San. A. (1955): 151–167.

[8] Madin, S. H., York, C. J., and McKercher, D. G.: Isolation of the Infectious Bovine Rhinotracheitis Virus. Science, *124*, (1956): 721.

[9] Dulbecco, R., and Vogt, M.: Plaque Formation and Isolation of Pure Lines with Poliomyelitis Viruses. J. Exptl. Med., *99*, (1954): 167.

[10] Earle, W. R.: Production of Malignancy *in Vitro*. IV. The Mouse Fibroblast Cultures and Changes Seen in the Living Cells. J. Nat. Cancer Inst., *4*, (1943): 165.

[11] Reed, L. J., and Muench, H.: A Single Method of Estimating Fifty Per Cent Endpoints. Am. J. Hyg., *27*, (1938): 493.

[12] Boyarnick, M. R., Miller, J. C., and Snyder, J. C.: The Influence of Certain Salts, Amino Acids, Sugars and Proteins on Stability of Rickettsiae. J. Bact., *59*, (1950): 509.

[13] Baker, J. A.: A Filterable Virus Causing Enteritis and Pneumonia in Calves. J. Exptl. Med., *78*, (1943): 435.

[14] Baker, J. A., York, C. J., Gillespie, J. H., and Mitchell, G. B.: Virus Diarrhea in Cattle. Am. J. Vet. Res., *15*, (1954): 525.

[15] Baker, J. A., and Little, R. B.: Leptospirosis in Cattle. J. Exptl. Med., *88*, (1948): 295.

[16] York, C. J., and Baker, J. A.: A New Member of the Psittacosis-Lymphogranuloma Group of Viruses that Causes Infection in Calves. J. Exptl. Med., *93*, (1951): 587.

The Cheek Teeth of Cattle

N. D. JONES, D.V.M., and L. E. ST. CLAIR, D.V.M., Ph.D.

Urbana, Illinois

STUDIES on teeth have usually placed emphasis on the individual tooth rather than its relationship to the others in series. When a tooth is studied as a member of a series or compared with its homologues in other species, certain trends become evident. Butler[1] stated that almost every detail on a tooth can be compared with corresponding details on adjacent teeth, and that only a common morphogenetic cause acting on more than one of the tooth germs could create such similarity. Thus, there is a gradation in form from tooth to tooth along the series. The series has differentiated into regions: incisor, canine, and molar, each of which has developed a degree of independence. Because of the length of the region and the greater number of teeth, morphological changes are most evident in the molar (cheek tooth) field.

Originally, the term "molar" was applied to teeth whose table surfaces were adapted to the grinding of food. All of the cheek teeth are considered members of the molar series, although the specific term, molar, has popularly been applied only to the posterior group. The temporary (milk, deciduous) and permanent molars are classified as primary cheek teeth. The premolars are members of the secondary series, since they replace the deciduous molars which also have often been referred to as deciduous premolars.[2] However, investigators, who have observed that the primary cheek teeth resemble each other more closely than they do the secondary ones, have labeled the temporary teeth deciduous molars.[3, 4]

The evolution of teeth toward the true molar pattern is called "molarization." Butler[5, 6] observed that in the primitive perissodactyla the premolars were less molariform than the deciduous molars which they replace. In the domestic perissodactyla (Equidae), molarization has progressed to a point where the premolars and deciduous molars are comparable. Molarization thus passes from the permanent molars to the deciduous molars and then to the premolars. The progressive chemodifferentiation is brought about in a way similar to the organization of any tissue.[7]

The standard number of premolars in mammals is four. Ruminants possess only three, however, which are usually referred to as upper or lower P1, P2, and P3. It has been postulated that the canine tooth has migrated to the area of the incisors, making them four in number, while the first premolar has moved to the canine position in those ruminants which possess a canine tooth.[8] On this basis, the premolars should be designated as upper or lower P2, P3, and P4. Since the deciduous molars are in the same position as the premolars, they also would be numbered 2, 3, and 4.

Discussions of the crowns of bovine cheek teeth have emphasized pulp canals,[5] lobes, folds, and capsules[4] but have generally omitted the cusps (prominences of the table surface). Homologous parts of cheek teeth in all mammals, as well as parts of adjacent teeth in a particular animal, can conveniently be described by reference to the cusps.[10]

An early discussion of artiodactyl teeth stated that the lower molars of ruminants are like the upper ones in reverse.[4] A recent paper by Garlick[9] gave a general description of the cheek teeth. He stated that the mandibular cheek teeth have two roots while the maxillary teeth have three roots. Small accessory roots are sometimes present in the former, rarely in the latter. He discussed fully the occlusion of the cheek teeth demonstrating their serrated appearance and their eccentric apposition.

Whereas eruption refers to the initial appearance of the tooth on the surface of the jaw, eruptive movements continue until the tooth is lost or the animal dies.[2] The growth of cementum and of the bone of the alveolus contributes to these movements.

In this paper, special emphasis will be placed on the morphology of cheek teeth based on cusps and on the extent to which

From the Department of Veterinary Anatomy and Histology, College of Veterinary Medicine, University of Illinois, Urbana.

molarization has progressed in cattle. In the many skulls examined, the variations in beef and dairy breeds of all ages were so slight that the general pattern was always retained.

OBSERVATIONS

The following symbols will be used to identify the individual cheek teeth: upper—dM^2, dM^3, and dM^4; P^2, P^3, and P^4; and M^1, M^2, and M^3; lower—dM_2, dM_3, and dM_4; P_2, P_3, and P_4; and M_1, M_2, and M_3.

The upper primary cheek teeth (dM^2, dM^3, and dM^4; M^1, M^2, and M^3) are similar in form with the exception of dM^2. The height of the crown is greater on the buccal than on the lingual edge. There are four major cusps arranged in anterior and posterior pairs or units (fig. 1 and 11a and b). The cusps of each unit are separated by an irregular, crescent-shaped infundibulum which is concave laterally. The convex medial surface of the lateral cusp and the concave lateral surface of the medial cusp form the infundibulum. The lateral edge of each lateral cusp forms a "W." The medial edge of each medial cusp forms a "U." The vertical groove on the medial surface of the tooth contains a pillar (accessory cusp), which extends from the neck to about 1 cm. from the occlusal surface of the unworn tooth. The crown of the unworn tooth is longer than it is wide at the occlusal surface and is wider than it is long near the root. The teeth increase in size gradually from the anterior to the posterior end of the series.

The crowns of the upper permanent molars are distinctly higher than those of the other cheek teeth and are irregularly rectangular in cross section. Each tooth has three roots placed so that one is beneath each lateral cusp and a wider one is situated medially (fig. 6). Each root is vertically grooved on the surface which faces the other roots. The wide root is grooved on its medial surface also. Deciduous M^3 is an exception to the pattern in that it has an anterior root extending from the anterior cusp unit and two posterior roots, one from each of the posterior cusps. Deciduous M^2 is a small, irregularly shaped, less complex tooth (fig. 1). The shallow infundibulum may be single or double. The edge of the crown is rounded anteriorly but pointed posterolaterally. Laterally, there is but one "W." There is no accessory cusp. The tooth has a straight

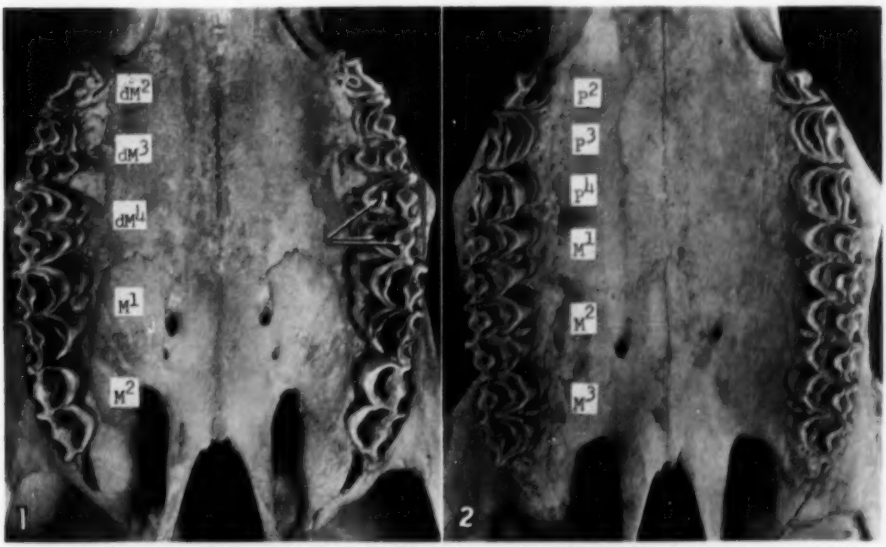


Fig. 1—Maxillary cheek teeth of beef heifer, 15 months old, showing deciduous molars and permanent molars. The last molar has not erupted. Arrows indicate the cusps.

Fig. 2—Maxillary cheek teeth of a Holstein-Friesian cow, 5 years old, showing premolars and permanent molars.

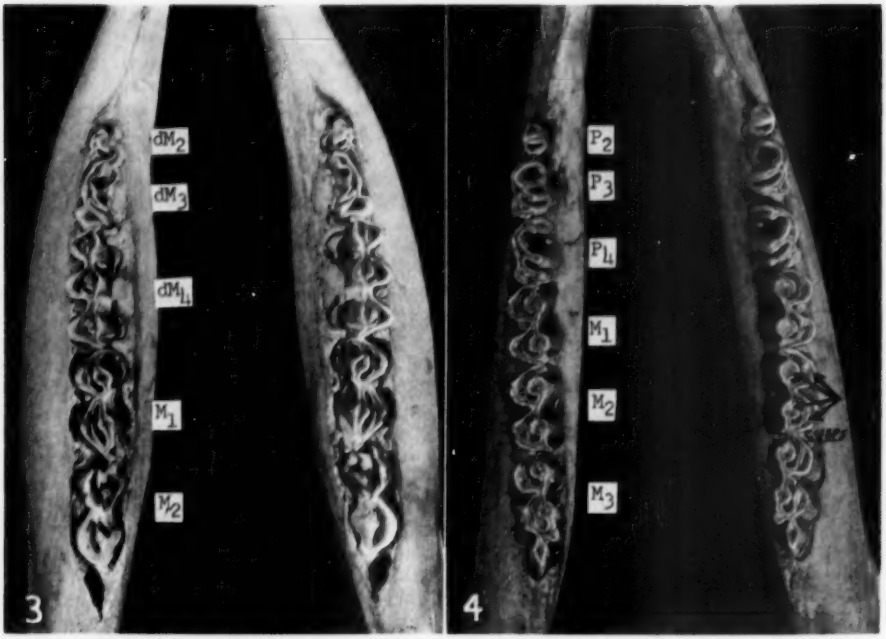


Fig. 3—Mandibular cheek teeth of a heifer, 15 months old (see fig. 1), showing deciduous molars and permanent molars. The last molar has not erupted.

Fig. 4—Mandibular cheek teeth of a cow, 5 years old (see fig. 2), showing the premolars and permanent molars. Arrows indicate the cusps.

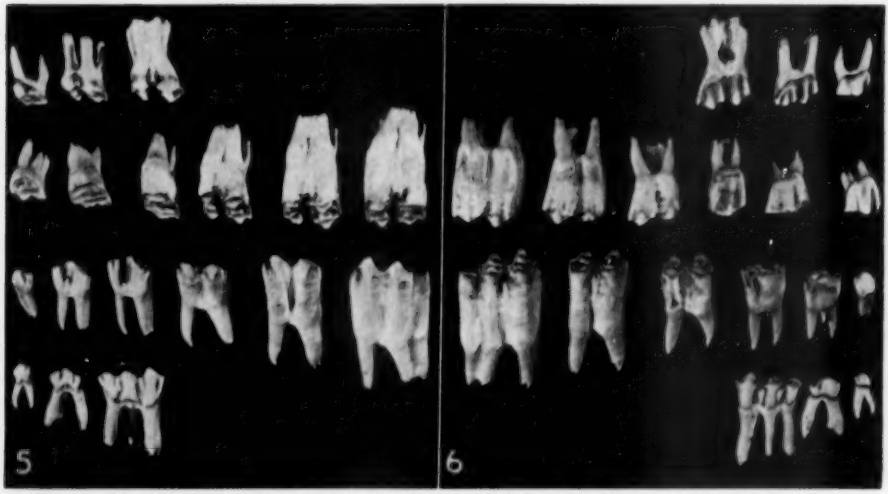


Fig. 5—Lingual view of the cheek teeth. Rows 1 and 4 are deciduous and 2 and 3 are permanent. The anterior teeth are on the left and the upper jaw is at the top.

Fig. 6—Buccal view of the cheek teeth. Rows 1 and 4 are deciduous and 2 and 3 are permanent. The anterior teeth are on the right and the upper jaw is at the top.

Fig. 7—Lateral view of the cheek teeth of a cow, 5 years old (see fig. 2 and 4), showing occlusion.

anterior root and a shorter, elbowed, posterior root. Each root is vertically grooved on the surface which faces the other root (fig. 5 and 6).

The upper premolars differ distinctly from the deciduous and permanent molars in the arrangement of cusps. The table surface of a premolar resembles closely a single individual cusp unit of the two identical ones possessed by a molar (fig. 2 and 11a, b, and c). The unit, however, is lengthened. The roots tend to follow the three-rooted pattern (fig. 5 and 6), although they are partially fused. Small accessory roots may be present. The crowns tend to be shorter than those of the permanent molars. Also they increase in size from P^2 to P^4.

The lower deciduous and permanent molars are in general similar to the upper ones except that they are narrower and reversed in design and form, *i.e.*, what is lateral in the lower jaw is medial in the upper jaw (fig. 11a, b, e, f, and g). Deciduous M_2 and dM_2 do not follow that design, however. Also in a typical lower cheek tooth there are two medial convexities per unit separated by a shallow vertical groove instead of two "W's" (fig. 3, 4, and 11f). Deciduous M_4 is unique in that it has three complete cusp units instead of two (fig. 3, 8, and 11e). The M_3 has a fifth cusp located posteriorly (fig. 4, 8, and 11g). A typical lower cheek tooth has two roots, one anterior and one posterior. The anterior root tends to be vertical, but the posterior one slopes slightly caudally (fig. 5, 6, and 9). In dM_4, there is also a slender root beneath the lateral cusp of the middle unit (fig. 5, 6, and 8). There may even be a medial rudimentary root. The posterior root of M_3 is large because of the fifth cusp (fig. 8). This root is triangular and grooved on its medial and lateral surfaces.

Deciduous M_3 is irregular in shape. The table surface is roughly triangular with the apex directed anteriorly (fig. 3 and 11d). The length of the crown is about twice the width. There are no infundibula

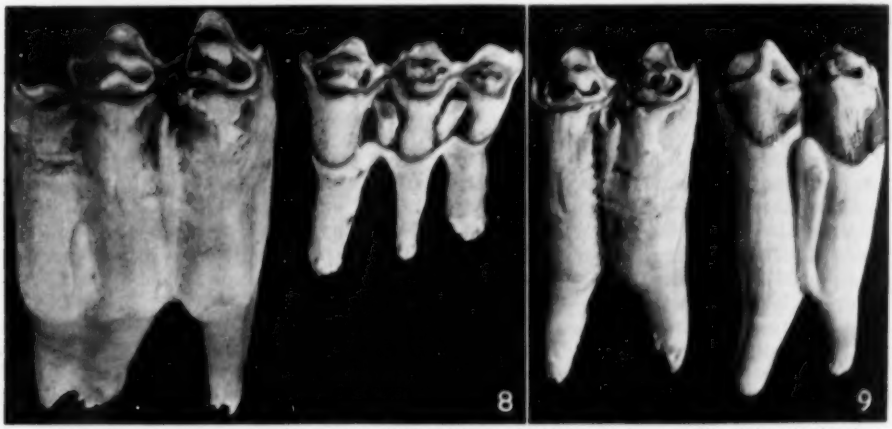


Fig. 8—Lower permanent molar 3 at the left and lower deciduous molar 4 at the right. Occlusal and buccal view.

Fig. 9—Lower permanent molar 2. The one on the left has been in wear longer than the one on the right.

Fig. 10—Upper permanent molar 2, showing cementum deposition.

but two grooves encroach upon the medial surface of the crown. A shallow groove is present about two thirds of the way back on the lateral surface. The two roots are farther apart near their apices. Deciduous M_2 is actually a simple, low-crowned, single-cusped tooth (fig. 3). The two roots are sometimes fused. Deciduous M_2 and the anterior two thirds of dM_3 differ also from the usual pattern in that the medial and lateral edges of the table surface may be equal in height, or the lateral edge may be slightly higher than the medial one. The anterior half of dM_2 does not contact an upper cheek tooth.

The differences between the lower premolars and the lower deciduous molars are not as great as is the case in the upper jaw, with the exception of dM_4 which was described above. The P_2 is like dM_2 except that it is larger. The P_3 and P_4 are similar to dM_3, except that their two medial grooves are more distinct (fig. 3 and 4). There may be a small infundibulum instead of the deep posterior groove on P_4.

The table surfaces of the cheek teeth as a group are serrated. The upper and lower teeth mesh so that the upper tooth is slightly posterior to the corresponding one of the lower set. Thus, one tooth contacts two opposing teeth. The lower first and upper last cheek teeth contact only one tooth each, however (fig. 7).

The height of the exposed portion of the permanent tooth remains relatively constant throughout the life of the animal (fig. 9). The continual wearing at the table surface makes it necessary that the eruptive movements be constant. The crown continues to get shorter. The roots, which are open at the apex, elongate until the root canal closes. Cementum is laid down on the apex of the roots and on the crown at the base of the roots. These processes go on indefinitely but may vary as to rate as is shown by the rings of cementum on the surface of the tooth (fig. 10). The alveolus becomes smaller by the proliferation of bone. Such processes affecting the deciduous teeth are transitory.

DISCUSSION

Inasmuch as Butler [5,6] found that molarization was complete in all of the cheek teeth in the modern horse, but that it had affected to a lesser degree the premolars and even the deciduous molars in the primitive horse, it is evident that molarization progresses forward through the deciduous molars and then the premolars. Although the cheek teeth of cattle are essentially grinders, complete molarization has progressed only through dM^3 and dM_4. While it is possible that the molarizing factors which influence the premolars are different from those affecting the other molars, the pattern in cattle seems to fit that of the primitive horse.

The permanent molars in some cases have been classified as secondary teeth and not primary teeth that erupt late.[11] The extra cusp unit on dM_4 and the extra posterior cusp on M_3 make these teeth somewhat similar in appearance (fig. 8 and 11e and g). Since each is the posterior tooth of its group, it was argued that the deciduous cheek teeth and the permanent molars are not closely related morphologically to each other. As has been shown by our investigation, these two teeth are ac-

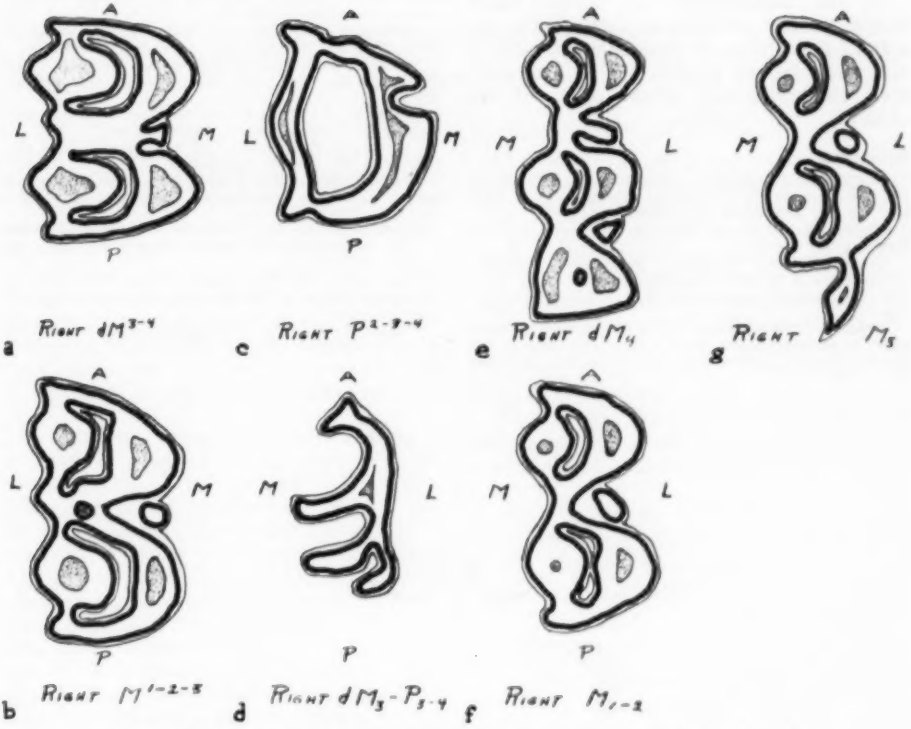


Fig. 11—Schema of the occlusal surfaces of representative cheek teeth of cattle.

tually not alike. It seems likely that the extra cusp unit on dM_4 is the anterior unit of that tooth rather than the posterior one, and represents the anterior end of the molarizing field.[12] The teeth that are replaced by the premolars, since they are thus in direct series with the molars, are more appropriately called deciduous molars than deciduous premolars.

The amount of tooth exposed beyond the gum line (which may recede slightly with age) is kept constant as attrition occurs by deposition of cementum on the roots and on the unexposed crown and by filling of the alveolus with bone, especially at its deepest portion.[4] The tooth substance is increased by the deposition of cementum at about half the rate at which it is lost by attrition. The proliferation of alveolar bone compensates for the other half.[4] Cementum is deposited most heavily at the apices of the roots and at the base of the crown between the roots. It apparently is deposited at varying rates, since in old teeth it appears in rings or steps. The masses of cementum formed at the apices of the roots are irregular in shape. Depressions on the crown are less distinct in older teeth because cementum tends to be deposited there more than on the prominences.

Summary

1) The form of the cheek teeth of cattle with special reference to cusp arrangement is described. The molar pattern has four major cusps set in pairs as anterior and posterior units.

2) "Molarization" proceeds serially in an anterior direction. In cattle, the end point of molarization has been achieved through dM^3 and dM_4. The other deciduous molars and the premolars are not typically molar in type. The process has advanced to a somewhat greater degree in the upper than in the lower deciduous molars. The premolars of both jaws are less molariform than the deciduous molars.

3) The maintenance of visible tooth height during attrition in cattle is the result of deposition of cementum in concentric rings on the hidden portion, particularly the roots, and of the filling of the alveoli.

References

[1] Butler, P. M.: Studies of the Mammalian Dentition. Differentiation of the Post-Canine Dentition. Proc. Zool. Soc. London, *109*, series B, (1939a): 1-34.

[2] Sicher, H.: Oral Anatomy. C. V. Mosby Co., St. Louis (1949): 242-276.

[3] Butler, P. M.: Studies of Mammalian Dentition. I. The Teeth of *Centetes Ecaudatus* and Its Allies. Proc. Zool. Soc. London, *107*, series B and C, (1937): 103-132.

[4] Owen, R.: Odontography: or, A Treatise on the Comparative Anatomy of the Teeth; Their Physiological Relations, Mode of Development, and Microscopic Structure in Vertebrate Animals. Text Vol. 1. Hippolyte Bailliere, London (1840-1845): 527-535.

[5] Butler, P. M.: The Milk Molars of Perissodactyla. Proc. Zool. Soc. London, *121*, (1951-1952): 777-817.

[6] Butler, P. M.: Molarization of the Premolars in the Perissodactyla. Proc. Zool. Soc. London, *121*, (1952): 819-843.

[7] Child, C. M.: Patterns and Problems of Development. University of Chicago Press, Chicago, 1941.

[8] Loomis, F. B.: Dentition of Artiodactyls. Bull. Geo. Soc. Am., *36*, (1925): 583–604.

[9] Garlick, N. L.: The Teeth of the Ox in Clinical Diagnosis. II. Gross Anatomy and Physiology. Am. J. Vet. Res., *15*, (1954): 385-394.

[10] Osborn, H. F.: The Evolution of Mammalian Molars to and from the Tritubercular Type. Am. Nat., *21*, (1888): 1067.

[11] Marston, A. T.: The Relation of the Deciduous and Permanent Molars in the Ox. Proc. Roy. Soc. Med., *31*, (1938): 495-496.

[12] Scott, W. B.: The Evolution of the Premolar Teeth in the Mammals. Proc. Acad. Sci. Phil., *44*, (1892): 405-444.

An Abnormality of the Bovine Penis

R. R. ASHDOWN, B.V.Sc., M.R.C.V.S.

Bristol, England

In 1906, Gerhardt [2] attempted to establish the relationships between the diverse forms of penis found in the Artiodactyla. He believed the basic plan to be that of a fibrous penis with a twisted and simple free end. This form is found in some living members of the order (e.g., Sus, *Tragulus meminna*). However, most forms now in existence possess a subterminal enlargement (which Gerhardt called an "accessory glans") and the distal part is modified to form a urethral process (fig. 1).

In the bovine penis, the enlargement is intimately attached to the urethra. The urethral process has shrunk to an insignificant papilla and the enlargement has, therefore, become terminal. The penis of the goat (Hircus) and sheep (Ovis) is less strongly modified from the basic plan represented by the Indian chevrotain *(Tragulus meminna)*. The blackbuck (Antilope) and the reedbuck (Cobus) show forms intermediate between ox and goat. Mandowsky [3] produced further evidence to support Gerhardt's theory. In particular, he showed that the urethral papilla of the ox is supported by fibrous bands much as the urethral process of the sheep and that, in the fetal ox, the urethra is less intimately fused to the rest of the penis and shows a marked degree of independence which is progressively lost as the animal becomes more mature. Böhm [1] also demonstrated the embryonic similarity between the urethral processes of oxen, sheep, and goats.

During an examination of over 500 penises from entire and castrated oxen of various ages, 1 case was encountered which showed an abnormality relevant to the theory outlined above. The animal was a castrated adult slaughtered at a Bristol abattoir in May, 1956. The penis was examined immediately after slaughter, photographed, and preserved in formalin.

Description of the Specimen

The penis was small and underdeveloped as is often found in castrated oxen. The free end (the part enclosed in the sheath when at rest) was 52 mm. long and the mean width at its base was 12 mm. The free end was separate from the sheath except for a small area at the base and the intact frenulum which stretched from the urethral process to the base of the free end. The frenulum was abnormally wide at the urethral process.

The general structure of the free end was normal; a conical part terminated in a terminal enlargement and a urethral ex-

Fig. 1—The free end of the penis of several ruminants, illustrating the relationships between the different types. (Diagrams modified from Gerhardt, 1906). The urethral process is shown in black and the terminal enlargement is stippled.

From the Department of Veterinary Anatomy, University of Bristol, England.

This work was done during the tenure of an A.R.C. research studentship, under the supervision of Prof. C. W. Ottaway. The material was photographed by Mr. A. Coombs.

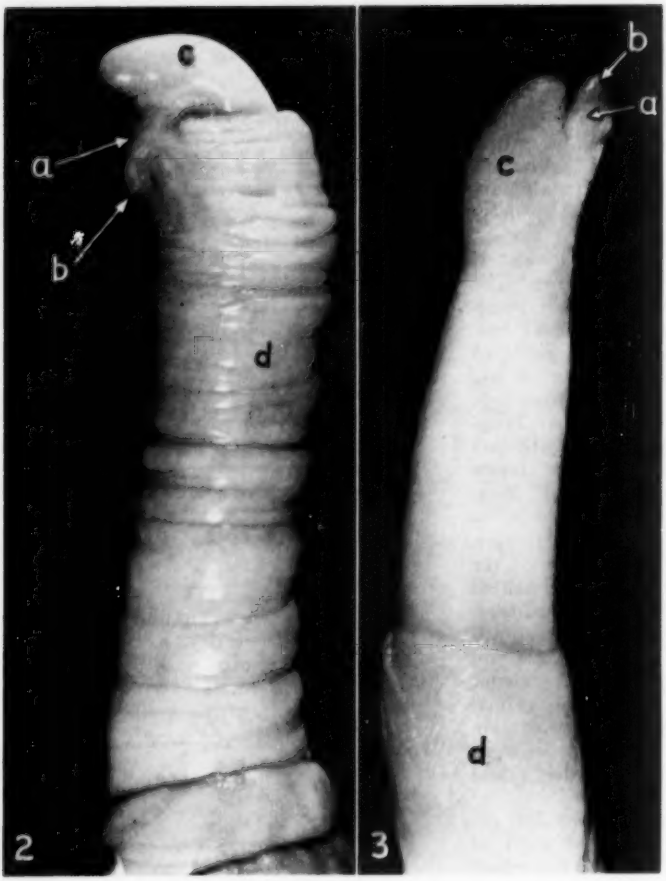


Fig. 2—The penis of a castrated ox, protruded from the sheath to show the abnormal arrangement at the apex (ventral view): (a) urethral process, adherent to the sheath; (b) urethral orifice; (c) terminal enlargement; and (d) reflected sheath.

Fig. 3—The same penis (fig. 2) of a castrated ox, with sheath separated from the penis and reflected, showing the bifid apex (dorsal view): (a) urethral process, separated from the sheath; b, c, d as in figure 2.

Fig. 4—The normal penis of a castrated ox with sheath reflected, showing the normal arrangement at the apex (ventral view): (a) urethral process; b, c, d as in figure 2.

tension; but the relationship between these two was abnormal. The urethral extension was well developed and separate from the terminal enlargement for a length of 10 mm. It formed a true urethral process which was adherent to the sheath (fig. 2). The urethra opened at the apical extremity of the urethral process, and the lumen of the urethra within the process was voluminous. The terminal enlargement was normal in shape and position but, because of its separation from the urethral process, the apex of the penis was bifid with the process projecting slightly in front of the enlargement (fig. 3).

For comparison, the normal free end of the penis of a castrated adult is shown (fig. 4). The terminal enlargement is recurved at its apex, encircling the urethral process to which it is fused so that only the short urethral papilla protrudes freely from the rest of the penis.

DISCUSSION

The intimate incorporation of the urethral process into the rest of the free end of the penis [3] appears not to have occurred in this specimen. The abnormality is not due to castration; nor are there indications that it is due to injury, for the degree of adhesion of penis to sheath is not unusual. It appears that the abnormality is congenital and might be found also in the entire bull, although no account of this has been found in the literature.

The form of penis found in this ox is strongly reminiscent of that seen in those members of the Artiodactyla in which a short thick urethral process exists (e.g., Antilope). This specimen illustrates the basic independence of the urethral process of the ox, and emphasizes the affinity of the bovine type of penis with that of related members of the order.

References

[1] Böhm, J.: Die äusseren Genitalien des Schafes. Morphol. Jahrb., *34*, (1905): 248.

[2] Gerhardt, U.: Zur Morphologie der Wiederkäuerpenis. Verhandl. d. deutsch. zool. Gesellsch., *16*, (1906): 149.

[3] Mandowsky, C.: Zur Morphologie und Histologie der Artiodactylenpenis. Ztschr. f. Anat. Entwicklungsgesch., *83*, (1927): 269.

Methods of Injecting Tuberculin in Cattle

AUBREY B. LARSEN, D.V.M., M.S.; LOUIS A. BAISDEN, Ph.D.;
RICHARD S. MERKAL, B.S.; MEREDITH J. MORRIS, B.S.

Auburn, Alabama

THE INTRADERMIC tuberculin test is widely employed to test millions of cattle each year. Although a standard procedure is used in conducting the tuberculin test in regard to the quality and quantity of tuberculin injected and the time that elapses between injection and observation, there is no standard procedure regarding the type of needle used or the method of inserting the needle to get the maximum response to the tuberculin injected. Lash [1] stated that the needle should be of the Schimmel type, 25-gauge, and preferably ¼ to ½ inch in length. He also stated that the needle should be inserted between the layers of skin, care being taken to inject neither too deeply nor too superficially. Johnson [2] stated that a 25- to 28-gauge needle, ⅜ inch in length, is most satisfactory. However, some veterinarians use a ⅛-inch, 22-gauge needle for making intradermic injections. The needle is thrust its full length into the skin when the injection is made.

The usual injection site is one of the caudal folds; however, in recent years, the skin in the neck region has been employed as a site in known tuberculosis-infected herds [3] because the skin in this region is more sensitive to tuberculin than the skin of any other region of the animal's body.[4] Effects of the site differences in the neck region have been reported by Wadley,[5] Larsen *et al.*,[4] Van Waveren,[6] and Sjollema,[7] but in no case was a critical evalution reported of these differences.

This paper presents results of experiments to evaluate three methods of injecting tuberculin in the caudal folds and the skin of the cervical region and to determine the site differences within the latter region.

MATERIALS AND METHODS

Six cattle, previously sensitized with a subcutaneous injection of killed *Mycobacterium tuberculosis* var. *bovis* suspended in mineral oil, received 20 intradermic injections each of 0.1 cc. of 1:2 dilution * of intradermic mammalian tuberculin; one injection in each of the caudal folds and nine on each side of the neck. The injections were made by three different methods: (1) as superficially intradermically as possible with a ⅜-inch, 27-gauge needle; (2) as deeply intradermically as possible without being subcutaneous, with the same needle; and (3) by thrusting a ⅛-inch, 22-gauge needle perpendicularly into the skin the full length of the needle. In making the injections with the ⅛-inch, 22-gauge needle, the needle was withdrawn a short distance before the fluid was injected. This was done because it was observed that, when the ⅛-inch needle was inserted full length into the skin and the tuberculin immediately injected, there was a tendency for some of it to leak back around the barrel of the needle. The skin of each injection site was measured before injection and 48 hours afterward. Measurements in millimeters were made with a dermic thickness gauge,[8] and increases in skin thickness were recorded as reaction size.

One month after the first test, 3 of these cattle were given a second subcutaneous injection of killed *Myco. tuberculosis* var. *bovis* in mineral oil to boost their sensitivity, because reactions on these 3 cattle were smaller than expected in the first test. A second test, identical to the first, was conducted on the same 6 cattle 60 days after the first test.

Two months after the second test, 5 of the 6 cattle again had their sensitivity boosted, this time by an intraperitoneal injection of killed *Myco. tuberculosis* var. *bovis*. This was followed with three more tests at 30-day intervals on the 5 resensitized animals. These tests were identical to the first and second tests except 0.2 cc. of 1:8 dilution of tuberculin was used.

Previous work [9] has shown that cattle sensitized with intraperitoneal injections of killed *Mycobacterium paratuberculosis* bacilli in oil may react to intradermic tuberculin injections and, since 5 such animals were available, they were also tested. They received three tests at 30-day intervals, identical to those described for the *bovis*-sensitized animals except that 0.2 cc. of 1:4 dilution of tuberculin was used.

The methods of injection on the caudal folds

From the Regional Animal Disease Research Laboratory, Agricultural Research Service, U. S. Department of Agriculture, Auburn, Ala.

From the Animal Disease and Parasite Research Division (Baisden) and Biometrical Services (Morris), ARS, U.S.D.A., Beltsville, Md.

* Diluted tuberculin was used throughout the experiment because undiluted tuberculin often causes large reactions with necrotic centers when injected into cattle sensitized with oil suspensions of killed *Myco. tuberculosis* bacilli.

followed an incomplete block design, identical sites being used on all animals. Pairings of any two of the three methods were made so that each method was paired twice with each of the others on a set of 6 animals and used an equal number of times on the right and left caudal folds. The placement of the three methods of injection on each side of the neck followed a three by three Latin square design. In this design, each method appears once in each row and once in each column (fig. 1), the rows and columns being rotated from animal to animal. The nine injections were placed 3 inches apart, the anterodorsal one being 3 inches behind the ear and 3 inches from the top of the neck.

RESULTS

An analysis of variance was not used on the data obtained from the injections made in the caudal folds because of failure to obtain reactions on a full set of 6 animals of each sensitivity as required by the design. An analysis of paired comparisons by the "t" test showed no significant differences between the three methods of injection. The average reactions on the caudal folds of *bovis*-sensitized animals were: superficial intradermic, 3.3 mm.; deep intradermic, 4.0 mm.; and thrust, 3.2 mm.; and of the *paratuberculosis*-sensitized animals: superficial intradermic, 1.9 mm.; deep intradermic, 2.4 mm.; and thrust, 1.9 mm.

The analysis of variance of the results of the injections made on the neck regions of the *bovis*-sensitized animals is shown (table 1); the average reactions are given at the bottom of the table. The first test was not used in this analysis because of failure to obtain reactions on 2 animals.

S—Superficial intradermic
D—Deep intradermic
T—Thrust

S	D	T
T	S	D
D	T	S

Fig. 1—An example of the placement of the methods of injection in a three by three Latin square design on the neck region.

There was a highly significant difference between the thrust and the two intradermic methods of injection. The rows and columns were significantly different; the lower and middle rows were different from the upper row, and the front and middle columns were different from the back column. The animals and tests were highly significantly different, but the sides of the neck, on the average, were not significantly different. The average results that were obtained in this series of tests might not be reproduced in another single trial or a test on a single animal but could be expected in testing several animals over a series of trials.

Several interactions (*i.e.*, method x animal, table 2) were significant. The meaning of this is elucidated by the presentation (table 2) of the ranking of the totals used in calculating the mean square of the variance due to the interaction of methods by animals. The numbers in parentheses refer to the rankings of the method totals for each animal. On 3 animals, the deep intradermic injection totaled over all tests ranked first and, on the other 2 animals, it ranked second. If the methods had ranked in the same order on all the animals, the interaction would probably not have been significant. The other interactions may be interpreted in a similar manner.

The analysis of variance of the results of

TABLE 1—Analysis of Variance and Average Reactions of Three Methods of Injection of Tuberculin on the Neck of Bovis-Sensitized Cattle

Source of variation	Degrees freedom	Sum of squares	Mean square
Rows	2	25.75	12.88*
Columns	2	26.08	13.04*
Methods	2	1,608.47	804.24†
Squares (Latin)	39	2,428.32	62.26†
Animals	(4)	(674.79)	168.7†
Sides	(1)	(0.32)	0.32 n.s.
Tests	(3)	(1,031.18)	343.73†
Animal x side	(4)	(44.51)	11.13*
Animal x test	(12)	(613.81)	51.15†
Other interactions	(15)	(63.71)	4.25 n.s.
Row x square	78	395.62	5.07 n.s.
Column x square	78	604.12	7.74†
Column x animal	(8)	(89.55)	11.19†
Column x side	(2)	(30.42)	15.21†
Column x test	(6)	(50.18)	8.36*
Other interactions	(62)	(433.97)	7.0†
Method x square	78	658.42	8.44†
Method x animal	(8)	(111.98)	14.0†
Method x test	(6)	(136.76)	22.79†
Other interactions	(64)	(409.68)	6.4*
Error	73	268.38	3.68
Totals	352	6,015.16	.....

Average reactions in millimeters

Methods		Rows		Columns	
Superficial intradermic	7.4	Lower	6.3	Front	6.3
Deep intradermic	7.7	Middle	6.2	Middle	6.2
Thrust	3.1	Upper	5.7	Back	5.7

* Significant at the 0.05 probability level. † Significant at the 0.01 probability level.
n.s. = not significant.

TABLE 2—Ranks of Reaction Totals Summed over Tests of the Methods of Injection for Each Bovis-Sensitized Animal

Methods	Animals					Totals
	1	2	3	4	5	
Superficial intradermic	196.0(1)*	210.4(2)	199.6(2)	129.4(1)	153.6(2)	889.0
Deep intradermic	183.8(2)	214.2(1)	231.6(1)	128.6(2)	168.2(1)	926.4
Thrust	42.2(3)	90.2(3)	141.6(3)	33.6(3)	63.0(3)	370.6
Totals	422.0	514.8	572.8	291.6	384.8	2,186.0

* Numbers in parantheses are relative ranks.

the injections on the neck regions of the *paratuberculosis*-sensitized animals is given (table 3); the average reactions are given at the bottom of the table. The row effects were not found to be significantly different

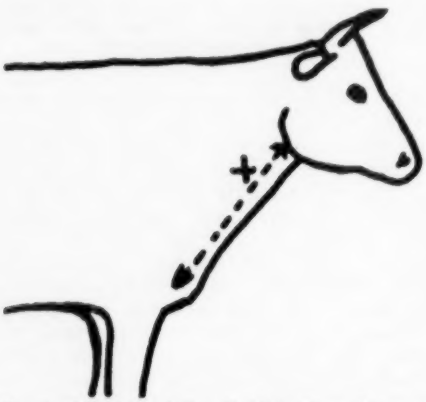


Fig. 2—Location of the site (x) of maximum reaction to tuberculin.

in the *paratuberculosis*-sensitized animals. Otherwise, the results were found to be similar to those obtained on the *bovis*-sensitized animals.

DISCUSSION

The results reported indicate that the deep intradermic method is the one of choice for general field testing on the neck region. It gave significantly larger reactions than did the thrust method and slightly larger reactions than the superficial intradermic method. Its use would appear to give more reliable diagnoses than would the use of the thrust method. More work would have to be done to prove that it is the method of choice on the caudal folds.

The results also suggest that the site of choice for maximum reactions for the injection of tuberculin is the anterior, lower point of the injection area on the neck. This site is located just above a line drawn from the angle of the mandible to the anterior extremity of the manubrium of the sternum. The site is approximately one-fifth the distance from the angle of the mandible to the manubrium. The animal must be in a normal position with both forelegs together (fig. 2).

It is puzzling that the thrust method gave nearly as large and as consistent reactions as the intradermic methods on the caudal folds, whereas this was not true on the neck. It is not known exactly the type of tissue into which the injections were made by the thrust method in either the caudal folds or the neck. Further it is not known the exact extent of back-leakage around the barrel of the needle which occurred by this method in either region. A fact of interest to be considered regarding

TABLE 3—Analysis of Variance and Average Reactions of Three Methods of Injection of Tuberculin on the Neck of Paratuberculosis-Sensitized Cattle

Source of variation	Degrees freedom	Sum of squares	Mean squares
Rows	2	7.85	3.93
Columns	2	15.66	7.83*
Methods	2	726.43	363.22†
Squares (Latin)	29	780.59	26.92†
Animals	(4)	(461.25)	115.31†
Sides	(1)	(0.02)	0.02 n.s.
Tests	(2)	(31.25)	15.62†
Animal x test	(8)	(245.19)	30.65†
Other interactions	(14)	(42.88)	3.06 n.s.
Row x square	58	157.22	2.71 n.s.
Column x square	58	195.28	3.37*
Method x square	58	296.43	5.11†
Method x animal	(8)	(56.58)	7.07†
Method x test	(4)	(41.78)	10.45†
Other interactions	(46)	(198.07)	4.31†
Error	60	127.74	2.13
Totals	269	2,307.20	.....

Average reactions in millimeters

Methods		Rows		Columns	
Superficial intradermic	5.1	Lower	4.4	Front	4.6
Deep intradermic	5.8	Middle	4.5	Middle	4.3
Thrust	2.0	Upper	4.1	Back	4.0

* Significant at the 0.05 probability level. † Significant at the 0.01 probability level.
n.s. = not significant.

the results obtained on the neck with the thrust method is that in 17 per cent of the cases it was observed that no reactions occurred, while marked reactions were observed in adjacent areas where the injections had been made by the other methods. In several cases, however, the thrust method gave as large reactions as did the intradermic injections.

SUMMARY

The effect of three methods—superficial intradermic, deep intradermic, and thrust—of injecting tuberculin has been studied in tests carried out on the caudal folds and neck regions of two groups of cattle. One group was sensitized by means of heat-killed *Mycobacterium tuberculosis* var. *bovis* and the second by heat-killed *Mycobacterium paratuberculosis*.

On the caudal folds, no significant differences in the results obtained by the three methods were observed. On the neck, however, the two intradermic methods gave significantly larger reactions than the thrust, the deep intradermic giving the largest.

On the neck region, the effect of site of injection was also studied, and it was found that, in general, the lower front sites gave the largest reactions.

References

[1] Lash, E.: Tuberculin Testing of Livestock. U.S. Dept. of Agric., Dept. Circ. 249, (1936): 17 pp.

[2] Johnson, H. W.: The Application of Research Findings in the Eradication of Bovine Tuberculosis. Proc. U.S. Livestock San. A. (1947): 261–266.

[3] Kuttler, A. K.: Report on Bovine Tuberculosis Eradication Project. Proc. U.S. Livestock San. A. (1948): 27–28.

[4] Larsen, A. B., Groth, A. H., and Johnson, H. W.: Allergic Response to Johnin and Tuberculin of Various Skin Regions of Cattle. Am. J. Vet. Res., *11*, (1950): 301–303.

[5] Wadley, F. M.: Experimental Design in Comparison of Allergens on Cattle. Biometrics, *4*, (1948): 100–108.

[6] Van Waveren, G. M.: Tuberculose-onderzoek bij runderen met P.P.D.-tuberculines. Tijdschr. Diergeneesk., *78*, (1953): 735–760.

[7] Sjollema, P.: Over aspecifiekereacties en hun oorzaken. Tijdschr. Diergeneesk., *78*, (1953): 761–772.

[8] Kummer, F. A., and Johnson, H. W.: Studies on Johnin. IV. Dermal Thickness Gauge. Am. J. Vet Res., *5*, (1944): 189–192.

[9] Johnson, H. W., Larsen, A. B., Henley, R. R., and Groth, A. H.: Studies on Johnin. VI. The relationship of the Allergens of *Mycobacterium Paratuberculosis*, *Mycobacterium Tuberculosis* var. *Avium*, *Bovis*, and *Hominis*, and *Mycobacterium Phlei*. Am. J. Vet. Res., *10*, (1949): 138–141.

An Acidified Antigen for Detection of Nonspecific Reactions in the Plate-Agglutination Test for Bovine Brucellosis

JOSEPH E. ROSE, Ph.D., and MARTIN H. ROEPKE, Ph.D.

Saint Paul, Minnesota

In 1951, Hess and Roepke [1] demonstrated the presence of a nonspecific factor in bovine serum that was responsible for some of the apparently false reactions in the plate-agglutination test for bovine brucellosis. They devised a filter-paper chromatographic test that would distinguish nonspecific reactions from the reactions resulting from contact or infection with organisms of the genus Brucella. In further work, Hess [2,3] isolated this factor and studied some of its properties.

Following the above studies, Amarasinghe [4,5] devised a second differential test based on the fact that selected rough strains of *Brucella abortus* would absorb nonspecific agglutinins from serum but would not absorb specific agglutinins.

In 1953, Hoerlein [6] reported the use of a 56 C. tube-agglutination test that would distinguish between specific and nonspecific agglutination reactions for brucellosis in swine serum. The nonspecific agglutinins were inactivated by heat at 56 C. for 18 hours. Morse *et al.*[7] applied this same technique to bovine serums.

The purpose of the present report is to describe another differential test which appears to distinguish between specific and nonspecific agglutinins according to their agglutination response at pH 4.0 in a plate-agglutination test.

A number of workers who studied the influence of low pH values on serological reactions have found that the response varies. The activity of antibodies might be lowered, destroyed, or even left unaltered, depending upon the system studied.

A low pH in the reaction mixture eliminated the precipitin reactions of the following antigens: diphtheria toxin,[8] egg albumin coupled with diazotized arsanilic acid,[9] egg albumin coupled with diazotized *m*-aminophenyltrimethyl-ammonium chloride,[10] and pneumococcus-specific soluble substance.[11]

In other cases, low pH merely diminished the activity of the antibodies, as in the absorption of antibody by red blood cells [12] and by bacteria.[13]

Finally, Kabat [14] found that a pH as low as 1.44 did not inactivate the antibodies to pneumococcus.

Experimental Procedures and Results

Preliminary Studies.—In the course of studies on the electrophoretic properties of purified nonspecific Brucella agglutinins prepared from serums by the method of Hess,[2,3] solutions of the purified preparations were dialyzed against buffers of various pH values. At times, the dialysis against buffers in the pH range of 4.0 to 5.0 diminished the agglutinating activity of the preparations.

On the hypothesis that the nonspecific agglutinins were acid labile, increasing amounts of 50 per cent acetic acid were added to 1.0-ml. volumes of a number of low-titer specific and nonspecific serums. The pH values were determined by a glass electrode pH meter, after which the agglutination titers were determined by the standard plate method. The agglutination tests showed that the titers were negative at pH values below 4.25 for the serums containing nonspecific agglutinins in contrast to the titers for the specific-type serums which either remained the same or else decreased one dilution at pH values between 3.8 and 4.25. The greatest difference in the agglutination titers for the two types of serums seemed to be in the pH range of 3.9 to 4.15 for the limited number of serums tested.

The encouraging results of these preliminary studies suggested a possible basis for a practical field test to distinguish between specific and nonspecific Brucella agglutination reactions. However, a simpler

From the School of Veterinary Medicine, University of Minnesota, St. Paul. Submitted as paper 3675, scientific journal series, Minnesota Agricultural Experiment Station, University of Minnesota.

Taken in part from a thesis submitted to the faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the Ph.D. degree. These studies were made possible by a grant from the Animal Disease and Parasite Research Branch, Agricultural Research Service, U. S. Department of Agriculture.

method than acidification of individual serums seemed desirable. Thus efforts were directed toward the development of an acidified antigen which would ensure a pH close to 4.0 for the serum-antigen mixture of the 1:25 dilution of the plate test.

TABLE 1—pH of Acidified Antigens and of Serum-Acidified Antigen Mixtures

	pH		
		Mixtures	
ml. concentrated acid/ml. of plate antigen	Acidified antigen alone	8 volumes serum, 3 volumes antigen	4 volumes serum, 3 volumes antigen
ACETIC (100%)			
0.01	3.84	5.1	.....
0.02	3.5	4.64	4.28
0.03	3.35	4.43	4.13
0.04	3.25	4.25	4.02
0.05	3.16	4.15	3.93
0.06	3.1	4.08	3.86
0.08	3.0	3.95	3.8
0.1	2.9	3.88	.....
0.14	2.73	3.75	.....
LACTIC (85%)			
0.03	2.68	4.02	.....
0.04	2.48	3.83	.....
0.06	2.33	3.6	.....
0.08	2.23	3.45	.....

Preparation of Acidified Plate Antigen (APA).—The antigen used was the "*Brucella abortus* antigen (plate method)" produced and distributed by the ARS, U.S. Department of Agriculture, for official use in the animal brucellosis control program in this country. The antigen contains 10.0 per cent (by volume) of heat-killed and washed *Br. abortus* cells in an aqueous solution of 0.5 per cent phenol and 0.85 per cent sodium chloride.

To determine the relationship between the amount of glacial acetic acid in the plate-test antigen and the pH of the serum-antigen mixture at the 1:25* dilution, increasing amounts of glacial acetic acid were added to constant volumes of antigen. Then, these acidified antigens were mixed with pooled, negative, bovine serum in the ratio of 8 parts of serum to 3 parts of antigen. In a few instances, the pH of the serum-antigen mixtures corresponding to the 1:50* dilution was also determined. The pH of these serum-antigen mixtures was measured by a glass electrode pH meter.

Since lactic acid also provides a good buffer system in the region of pH 4.0, similar measurements were made with lactic acid.

In the type of results obtained in this study (table 1), the data show that 0.06 to 0.08 ml. of glacial acetic acid per milliliter of plate antigen provides a good buffer system at the 1:25 dilution of the serum-antigen mixture. Appreciably less lactic acid is needed to obtain the same pH.

Attempts were made to develop a more highly buffered antigen by trials with various buffers composed of organic acids and their sodium salts. Such an antigen has two main advantages. The pH values of the 1:25, 1:50, and 1:100 dilutions would differ less from one another than they differ when a single acidified, nonbuffered antigen is used. Furthermore, the Brucella cells would not be exposed to as low a pH in a buffered antigen as that in a nonbuffered one. Hence, less deterioration of the cells should occur on standing. For these studies, the Brucella cells were centrifuged out of plate-test antigen and the supernatant fluid was replaced with either 1 *m* acetate, 2 *m* acetate, 1 *m* lactate, or 1 *m* citrate buffer. Appropriate acid-salt ratios to provide a pH of 4.0 of the serum-antigen mixture for the 1:25 dilution of the plate test were selected.

These more highly buffered antigens, in most instances, differentiated between specific and nonspecific reacting serums, but the results were less satisfactory than with the antigens acidified with the acids alone. In some instances, the higher salt concentrations in the more highly buffered antigens caused the serum-antigen mixture to jell. In addition, these antigens were occasionally agglutinated by serums containing nonspecific agglutinins or even by some of the negative serums.

The above pH studies were made with pooled negative serum obtained from samples submitted to the Animal Diagnostic Laboratory, University of Minnesota, for routine Brucella agglutination tests. A question arises as to whether the buffer capacity or acid-binding capacity of individual serums might vary sufficiently to result in appreciable variations in the pH of the serum-antigen mixture. To obtain information on this point, 1.0-ml. quantities of five different serums were acidified with 0.01 *n* hydrochloric acid and the pH

* Serum quantities of 0.08, 0.04, and 0.02 ml. with 1 drop (0.03 ml.) of antigen are, for convenience in this report, designated as 1:25, 1:50, and 1:100 dilutions, respectively, for the plate-agglutination test.

changes followed with a glass electrode pH meter. The amount of 0.01 *n* HCl required to reduce the pH to 4.0 was 7.0 ± 0.75 ml. The titration data also showed that the buffer capacity of serum in the range of pH 3.8 to 4.2 is nearly twice that in the pH range of 4.8 to 5.2. To obtain additional data, 7.0-ml. amounts of 0.01 *n* HCl were added to 1.0-ml. volumes of 19 randomly selected bovine serums and the pH values of the mixtures determined. The pH values ranged from 3.88 to 4.17, with an average of 4.01.

The serums from different animals appear to be fairly uniform in their buffer capacity and acid-binding capacity. The relatively large amount of acetic acid added to the plate antigen provides a buffer system which maintains the pH of the serum-antigen mixtures at the 1:25 dilution within ± 0.05 pH units of the average for individual serums. Variations of this degree for serum-antigen mixtures for individual serums lie well within the pH range of 3.9 to 4.1, which the limited laboratory studies indicate as the optimum pH range for inhibition of nonspecific agglutination reactions as compared with specific reactions.

The buffer capacity or acid-binding capacity of the plate antigen is appreciably lower than that of serum. Approximately 0.006 ml. of glacial acetic acid is required to lower the pH of 1.0 ml. of plate antigen to pH 4.0, whereas 0.023 ml. of acid (nearly 4 times as much) is required to lower the pH of 1.0 ml. of serum to the same point. The pH of the serum-antigen mixture therefore depends largely on the volume of serum present, since the volume of antigen used (0.03 ml.) for each dilution of the plate test is constant.

For the initial phases of a field trial with the acidified plate-test antigen, a separate acidified antigen was used for each of the first three dilutions of the test. The antigens for the 1:50 dilution and the 1:100 dilution contained one-half and one-fourth as much acetic acid, respectively, as the antigen for the 1:25 dilution. All three dilutions (1:25, 1:50, 1:100) had approximately the same pH.

Mechanism of Action of the Acidified Plate-Test Antigen.—The low pH value (4.0) of the serum-antigen mixture causes an inhibition of the nonspecific agglutination reactions rather than inactivation or destruction of the nonspecific agglutinins. This may be demonstrated easily by adding a drop of a saturated solution of sodium carbonate to the nonspecific serum and antigen mixture after the 15-minute incubation period. Agglutination follows rather quickly after neutralization of the acetic acid.

Comparison of Acidified Plate Antigen Test with the 56 C. Tube Test of Hoerlein.[6]—The serums used to compare the two tests were of three kinds: (1) serums largely in the suspect titer range selected at random from those submitted to the Animal Diagnostic Laboratory, University of Minnesota, for routine Brucella agglutination tests; (2) serums from animals purchased for sources of serum for the isolation and purification of nonspecific agglutinins—these animals were selected on the basis of nonspecific agglutination reactions to one or more of the three tests (the filter-paper chromatographic test,[1] the 56 C. tube test,[6] or the absorption test with a rough strain of Brucella[5]) mentioned earlier in this report; and (3) serums prepared by the addition of purified nonspecific agglutinins to pooled negative serum in amounts sufficient to raise the titer to the 1:50 to 1:100 range. The agglutinins were isolated from the serums of a number of animals mentioned above (2).

The test for heat-labile, nonspecific agglutinins was, with a slight modification, that described by Hoerlein[6] for swine serum. The antigen used was the official tube-test antigen prepared and distributed by the ARS, U.S.D.A. This antigen contains 0.045 per cent heat-killed cells of *Br. abortus* in an aqueous solution of 0.5 per cent phenol and 0.85 per cent sodium chloride. The tests were prepared in duplicate, beginning at the 1:25 dilution (0.08 ml. of serum). The serum was pipetted into the dry tubes (13 by 100 mm.) and the antigen (2.0 ml.) was added later. For the 56 C. test, it was found desirable to place the tubes in the 56 C. water bath immediately after adding the antigen. Any significant delay in this step appeared to cause inconsistent results. A 56 C. air incubator was not satisfactory, apparently because of the time required for the test mixture to reach the desired temperature of 56 C.

Following incubation in the water bath for 18 hours, the tests were not read di-

rectly, as recommended by Hoerlein, for in some instances, the 56 C. heat test produces inhibitors which prevent the formation of agglutination clumps that are large enough to be visible or to settle to the bottom of the tubes. The tests are then difficult to read and, in some instances, false negative reactions are obtained. Hence, Hoerlein's procedure was modified to include centrifugation of the tubes, after incubation, at 2,000 r.p.m. (radius of 17 cm.) for five minutes; decantation of the supernatant fluid; and replacement of the fluid by saline (0.85% NaCl). This added step before reading the tubes eliminates reading difficulties due to heat-produced inhibitors.

TABLE 2—Comparison of Tube Test (56 C. for 18 Hours) with Acidified Plate-Antigen (APA) Test on Selected Field Serums from Nonvaccinated Suspect Animals

Results of tube test (56 C. for 18 hr.)	APA test results, pH 4.0	No. of serums
−	−	32
−	+	1
+	−	16
+	+	31
Total		80

− = no agglutination; + = complete agglutination.

The replicate tube test was incubated at 37 C. for 48 hours in an air incubator to serve as a standard control test.

The procedure for the differential plate test was as follows: the test was prepared in duplicate with 0.08, 0.04, and 0.02 ml. of the serum pipetted onto the glass plate for the 1:25, 1:50, and 1:100 dilutions, respectively. One drop (0.03 ml.) of the standard plate antigen was added to each quantity of serum for the control test, and 1 drop (0.03 ml.) of the acidified plate-test antigen (0.08 ml. of glacial acetic acid/ml. of antigen) was added for each dilution of the acidified test. The serum and antigen for each dilution were mixed with a wire loop, starting at the 1:100 dilution. The "spot diameters" for the serum-antigen mixtures were approximately 21, 25, and 31 mm. for the 1:100, 1:50, and 1:25 dilutions, respectively. After mixing with the wire loop, the plate was rotated briefly to provide additional mixing and was allowed to stand at room temperature for 15 minutes, with rotation of the plate at seven or eight minutes to assist in bringing the reaction to completion. After 15 minutes' standing at room temperature, the plate was rotated again and the test then read. The reactions were recorded as "+" for complete agglutination, "I" for incomplete agglutination, and "—" for no agglutination for each dilution of the test. For the 1:25 dilution with the acidified antigen, trace reactions were also recorded as incomplete agglutination and interpreted as positive for specific agglutinins.

A summary of the results obtained on 80 selected field serums from nonvaccinated suspect animals is shown (table 2). Of the 80 serums tested, 33 (41.3%) were negative to the 56 C. tube test, indicating the presence of nonspecific agglutinins,

TABLE 3—Comparison of the Tube Test (56 C. for 18 Hours) with the APA Test on Nonspecific Reacting Serums and on Purified Nonspecific Agglutinin Added to Pooled Negative Serum

Serum No.	Tube test		Plate test	
	Control 37 C. for 48 hr.	56 C. for 18 hr.	Control	APA test
Nonspecific Reacting Serums				
374	+ + − − −*	+ + + − −	+ + I − −	− −†
846	+ − − − −	+ − − − −	+ + I − −	− −
883	+ − − − −	− − − − −	+ I − − −	− −
1198	+ + + + −	− − − − −	+ + + + −	I −
1199	+ + + + −	+ − − − −	+ + + + +	− −
1200	+ + + I −	+ + − − −	+ + I − −	− −
1219	+ + + + −	+ + − − −	+ + + I −	− −
Purified Nonspecific Agglutinins Added to Negative Serum				
41	+ + − − ...	+ I − − −	+ I − − −	− −
547	+ + + − −	− − − − −	+ + − − −	− −
374	... ...	... ...	+ − − − −	− −
1198	... ...	... ...	+ + I I −	− −
1199	... ...	... ...	+ + I I −	− −
1200	... ...	... ...	+ + − − −	− −

* Dilutions of 1:25 to 1:400; + = complete agglutination, I = incomplete agglutination, and − = no agglutination.

† Dilutions of 1:25 and 1:50.

whereas 48 (60.0%) were negative to the APA test. About 45.0 per cent more negative tests were obtained with the APA test than with the 56 C. tube test.

A summary of the results obtained on serums from animals which were purchased as sources of serum for the concentration and purification of nonspecific agglutinins is shown (table 3). Included in the tests were "synthetic" serums prepared by adding small amounts of the purified nonspecific agglutinin preparations to pooled negative serum.

The same type of differences between the results of the two different tests was obtained with the special serums as occurred with the selected field serums.

All serums, from which nonspecific agglutinins were isolated and purified in high yield by the method of Hess,[8] showed negative reactions to the APA test. The purified nonspecific agglutinins, when dissolved in negative serums, were again inhibited by the acidified antigen.

Apparently, both heat-labile (56 C. for 18 hours) and heat-stable forms of nonspecific agglutinins occur. The dual nature of these agglutinins is confirmed by studies of their physicochemical properties which show that two types of nonspecific agglutinin exist, corresponding to the differences in response to the 56 C. test.[15] One type has an isoelectric point of pH 4.32 to 4.35 and is inactivated at 56 C. The second type has an isoelectric point of pH 4.7 to 4.73 and is stable at 56 C. for 18 hours. The isoelectric point of bovine *gamma* globulin under the same conditions is pH 5.9 to 6.0. All isoelectric pH points were determined by microelectrophoresis. The physicochemical studies on the purified nonspecific agglutinins will be described in a future report.

DISCUSSION

The preliminary studies reported herein on the effect of pH on the agglutination reactions for Brucella indicate that an acidified plate-test antigen might serve as a practical field test to distinguish between specific and nonspecific Brucella agglutination. Such a test is being used on a large number of low titer serums from the field to determine its reliability under field conditions.

There are no obvious explanations of why agglutination of Brucella by nonspecific agglutinins is inhibited by pH values in the region of 4.0, whereas reactions with specific agglutinins usually remain unchanged. The reduction of the pH from approximately 7.4 (normal serum) to pH 4.0 reverses the electrostatic charge on both the specific and nonspecific agglutinins from negative to positive. If the antigen combines more firmly with the specific than with the nonspecific agglutinin, it is conceivable that such a reversal of electrostatic charge might loosen the bond between antigen and nonspecific agglutinin while leaving unchanged the bond between antigen and specific agglutinin.

The differences in the results obtained with the 56 C. tube test and the APA test on serums, as well as the studies on the isoelectric point determination on purified preparations of nonspecific agglutinins, indicate that there are at least two types of nonspecific agglutinins. A casual inspection of the preliminary results of the field study with the acidified plate antigen suggests that there may be other types of nonspecific agglutinins. This possibility needs to be investigated.

Attempts were made to extend the studies to a tube test at pH 4.0. The tube-test antigen used was suitably buffered with a sodium acetate-acetic acid buffer. At pH 4.0, such large amounts of serum proteins precipitated during the 48-hour incubation period at 37 C. that any agglutination reaction would not be observed.

SUMMARY

1) At pH values in the region of 4.0 for serum-antigen mixtures of the plate test, nonspecific Brucella agglutination reactions are inhibited, whereas the specific reactions remain unchanged or are but moderately inhibited.

2) To achieve a pH of 4.0 on the plate test, use is made of an acidified antigen prepared by adding 0.08 ml. of glacial acetic acid to 1.0 ml. of *Brucella abortus* antigen (plate method). When 0.03 ml. of this acidified antigen is added to 0.08 ml. of serum, as is done at the 1:25 dilution of the plate test, the resulting mixture has a pH of 4.0. Preliminary results indicate that this acidified plate antigen can distinguish between specific and nonspecific Brucella agglutinins.

3) The mechanism of action of the acidified antigen is one of inhibition of the agglutination reaction rather than of an inactivation or destruction of the nonspecific agglutinins as shown by the finding that neutralization of the serum-antigen mixture restores the agglutinating activity of the serum.

References

[1] Hess, W. R., and Roepke, M. H.: A Non-Specific Brucella-Agglutinating Substance in Bovine Serum. Proc. Soc. Exptl. Biol. and Med., *77*, (1951): 469-472.

[2] Hess, W. R.: Studies on a Nonspecific Brucella-Agglutinating Substance in Bovine Serum. I. The Differentiation of the Specific and Nonspecific Agglutinins by Heat Treatment. Am. J. Vet. Res., *14*, (1953): 192–194.

[3] Hess, W. R.: Studies on a Nonspecific Brucella-Agglutinating Substance in Bovine Serum. II. Isolation and Purification of the Brucella-Agglutinating Substances. Am. J. Vet. Res., *14*, (1953): 195–197.

[4] Amarasinghe, A.: New Procedures for Differentiating Specific from Non-Specific Agglutination in Bovine Brucellosis Serum and Milk Tests. I. Absorption Studies of Suspicious Bovine Serums with 9R Rough Antigens. Arch. Inst. Pasteur, Tunis, *32*, (1955): 259–279; French abstr., 280–282.

[5] Amarasinghe, A.: New Procedures for Differentiating Specific from Nonspecific Agglutination in Bovine Brucellosis Serum and Milk Tests. II. A Differential Test for Separating the Nonspecific from the Specific Agglutination in Suspicious Titered Serums. III. A Differential Procedure for the Separation of "False" Positive from "True" Positive Milk Ring Tests. Arch. Inst. Pasteur, Tunis, *32*, (1955): 491–518; French abstr. 519–522.

[6] Hoerlein, A. B.: Studies on Swine Brucellosis. III. The Differentiation of Specific and Nonspecific Agglutination Titers. Cornell Vet., *43*, (1953): 28–37.

[7] Morse, E. V., Schneider, D. W., and McNutt, S. H.: The Effect of Incubation at 56 C. on the Tube-Agglutination Test for Bovine Brucellosis. Am. J. Vet. Res., *16*, (1955): 269–273.

[8] Schmidt, S.: Ueber den Einfluss verschiedener Elektrolyten auf die Stabilitats-und Neutralisationsverhaltnisse des Diphtherietoxins und des antidiphtherischen Serums. Die Bedeutung der Elektrolyten fur die Reaktion zwischen Toxin und Antitoxin. Ztschr. f. Immunitätsforsch. u. exper. Therap., *67*, (1930): 197-239.

[9] Ito, M., Moriyama, T., and Sumiyoshi, S.: Studies on the Mechanism of the "Acid Turbidity Reaction." II. Analysis of the Combination between Azo-protein Antigen with Negatively Charged Determinant Groups and Its Homologous Antiserum in Acid Media. M. J. Osaka Univ., *5*, (1954): 311–316.

[10] Ito, M., Moriyama, T., and Sumiyoshi, S.: Studies on the Mechanism of the "Acid Turbidity Reaction." III. Analysis of the Combination Between Azo-protein Antigen with Positively Charged Determinant Groups and Its Homologous Antiserum in Acid Media. M. J. Osaka Univ., *5*, (1954): 317–322.

[11] Sobotka, H., and Friedlander, M.: The Precipitin Reaction of Antipneumococcus Sera. I. The Precipitin Index. J. Exptl. Med., *47*, (1928): 57–78.

[12] v. Euler, H., and Brunius, E.: Studien uber die Bindung Hämolytischer Antikörper. Ztschr. f. Immunitätsforsch. u. exper. Therap., *72*, (1931): 65–92.

[13] De Kruif, P. H., and Northrop, J. H.: The Stability of Bacterial Suspensions. IV. The Combination of Antigen and Antibody at Different Hydrogen Ion Concentrations. J. Gen. Physiol., *5*, (1922): 127–138.

[14] Kabat, E. A.: The Molecular Weight of Antibodies. J. Exptl. Med., *69*, (1939): 103–118.

[15] Rose, J. E.: A Study of Non-Specific Brucella Agglutinins in Bovine Serum. Ph.D. thesis, University of Minnesota, 1955.

Interference Between Anaplasmosis and Eperythrozoonosis in Splenectomized Cattle

LON E. FOOTE, D.V.M.; HELEN E. LEVY, B.S.; BETTY J. TORBERT, B.S.; W. T. OGLESBY, D.V.M., M.S.

Baton Rouge, Louisiana

VARIOUS INVESTIGATIONS have demonstrated that under given conditions some viruses, either inactivated or infectious, will interfere with the simultaneous multiplication of other viruses in the same host.[1-12] In some instances, the interference has proved to be reciprocal.[6,7,13-16] It has also been demonstrated that two viruses capable of reciprocal interference may simultaneously infect the same host.[17,18] Interference has been demonstrated between Bartonella and Eperythrozoon infections in mice.[19]

Anaplasmosis is generally considered to be a protozoan disease caused by *Anaplasma marginale*, a body which appears on or near the periphery of erythrocytes. However, this concept has been challenged.[20] The Anaplasma body does not "behave" like the causative agent of the disease, but characterizes itself as an inclusion body.[21]

Eperythrozoonosis is a rickettsial disease, the causative agent of which is readily filtered through ultrafine fritted glass filters or diatomaceous filters of fine porosities.[22-24]

MATERIALS AND METHODS

This report includes recent anaplasmosis research and a review of the anaplasmosis research records at the Louisiana Experiment Station dating from November, 1943. The calves employed in this research, a total of 197, were purchased from auction sales, the University beef and dairy herds, and from local stockmen. These animals were splenectomized at the ages of 4 to 205 days. Blood for inoculation purposes was taken from various anaplasmosis-carrier animals. Sodium citrate was used as an anticoagulant. The blood was administered to the calves subcutaneously or intravenously in amounts varying from 2 to 500 ml.

Blood samples were taken routinely from the experimental calves three times weekly. Some studies necessitated that samples be obtained daily. Blood film examinations were made of each blood sample. Total red and white blood cell counts, hemoglobin, and hematocrit determinations were made on alternate days.

From the Department of Veterinary Science, Louisiana State University, Baton Rouge.

DISCUSSION

Bovine eperythrozoonosis and anaplasmosis are two separate and distinct disease entities of splenectomized cattle. Evidently, the former is widespread in Louisiana but either does not cause clinical signs in nonsplenectomized cattle or perhaps such signs do occur but have not been properly interpreted. However, anaplasmosis is a recognized infectious disease of nonsplenectomized cattle. On different occasions, eperythrozoonosis has occurred spontaneously in baby calves after splenectomy. The clinical signs of the acute or peracute form of this disease in splenectomized calves are rectal temperatures ranging from 102.8 to 106.0 F., inappetence, depression, and acute muscular weakness. The total red blood cell count in acutely sick calves may vary from 3,500,000 to 6,000,000 per cubic millimeter. However, animals sick with anaplasmosis show weakness only when they become acutely anemic. Eperythrozoonosis appears to be easily transmitted between calves by flies or possibly by direct contact. This disease continues to be a problem in anaplasmosis research at the Louisiana Station where the laboratory animal most frequently employed is the splenectomized calf.

When 5 ml. of eperythrozoonosis-free anaplasmosis inoculum is administered to eperythrozoonosis-free, anaplasmosis-susceptible splenectomized calves either subcutaneously or intravenously, the average incubation period is 14 days (from date of inoculation to the first rise in body temperature).

From October through December, 1953, 5 calves were each inoculated with 5 to 250 ml. of blood from 1 of 5 different adult nonsplenectomized anaplasmosis carriers. Calf 69913 received inoculum from 2 anaplasmosis carriers, 1 of which was nonsplenectomized (table 1). Three of these carriers, 69597, 80224, and 69596, had recovered from experimental anaplasmosis. The other three, F–1, F–2, and F–3, had field cases

TABLE 1—Eperythrozoonosis Interference with Anaplasmosis Incubation Periods

Splenectomized calf No.	First inoculation			Second inoculation			Termination
	Date	Source	Amount	Date	Source	Amount	
69908	11–10–53	F–2	100 ml.	—	—	—	Destroyed 22 days after inoculation because of eperythrozoonosis.
69909	11–3–53	F–1	60 ml.	—	—	—	Destroyed 29 days after inoculation because of eperythrozoonosis.
69910	11–3–53	69597	50 ml.	—	—	—	Destroyed 29 days after inoculation because of eperythrozoonosis.
69913	12–4–53	80224	250 ml.	1–13–54	37177	250 ml.	Mild eperythrozoonosis followed by peracute anaplasmosis. Died 58 days after first inoculation.
69914	12–9–53	69596	5 ml.	—	—	—	Mild eperythrozoonosis followed by peracute anaplasmosis. Died 60 days after inoculation.
69106	12–21–53	F–3	250 ml.	—	—	—	Acute anaplasmosis (27 days incubation period) followed by eperythrozoonosis. Destroyed 40 days after inoculation because of eperythrozoonosis.

Note.—When microscopic findings or clinical signs show a calf to have eperythrozoonosis, the calf is either destroyed or turned to pasture for further observation.

of acute anaplasmosis. Blood was taken for inoculation studies from F–1 and F–2 within 24 hours after these animals were given 1 Gm. of oxytetracycline hydrochloride intravenously. This small dose of antibiotic is suppressive to the anaplasmosis etiological agent, as evidenced by the fact that splenectomized calves inoculated at this time from F–1 and F–2 developed eperythrozoonosis but not anaplasmosis. Blood was taken from F–3 for inoculation studies two weeks after this animal had been given 1 Gm. of oxytetracycline hydrochloride intravenously. By this time, the anaplasmastatic effect of the antibiotic evidently had been dissipated, since the calf inoculated with this blood developed acute anaplasmosis followed by eperythrozoonosis. None of these calves developed anaplasmosis within the usual 14-day incubation period (table 1). Because of this phenomenon, a review of the anaplasmosis research records at the Louisiana Station was made the last part of December, 1953.

From November, 1943, to October, 1945, 10 calves were each given 5 ml. of anaplasmosis inoculum from 1 of 4 carriers. The anaplasmosis incubation periods of these calves varied from 14 to 38 days. From October, 1945, to January, 1948, 15 calves were inoculated from 1 splenectomized cow, 37177, which was a carrier of both anaplasmosis and eperythrozoonosis. The inoculum dosage varied from 3 to 250 ml. The anaplasmosis incubation period of these calves ranged from six to 23 days. Those receiving the larger amounts had the shorter incubation periods. From November, 1950, through January, 1953, 95 calves were each given 5 ml. of inoculum from cow 37177. The anaplasmosis incubation periods of these calves were prolonged and varied from 19 to 61 days.

Cow 37177 developed a recrudescent acute attack of eperythrozoonosis the latter part of February, 1953. Seven calves were given 5 ml. of blood from cow 37177 from March 29 to April 20, 1953 (table

TABLE 2—Acute Eperythrozoonosis Infections Resulting in Altered (Prolonged) Anaplasmosis Incubation Periods

Splenectomized calf No.	First inoculation			Second inoculation			Days to development of anaplasmosis after first inoculation
	Date	Source	Amount	Date	Source	Amount	
69592	3–30–53	37177	5 ml.	6–11–53	69597	5 ml.	100
69202	4–2–53	37177	5 ml.	6–11–53	69597	5 ml.	94
69204	4–7–53	37177	5 ml.	6–11–53	69597	5 ml.	92
69223	4–7–53	37177	5 ml.	6–11–53	69597	5 ml.	80
69224	4–9–53	37177	5 ml.	6–11–53	69597	5 ml.	69
69208	4–20–53	37177	5 ml.	6–11–53	69597	5 ml.	59
69211	4–20–53	37177	5 ml.	6–11–53	69597	5 ml.	79
69912	12–3–53	69596	200 ml.	9–21–54	69596	5 ml.	Recovered from acute eperythrozoonosis and appeared normal clinically until sickening with peracute anaplasmosis after the third inoculation. Died 339 days after the first inoculation.
				*10–8–54	96102	10 ml.	

* Third inoculation.

2). None of these calves sickened with anaplasmosis when expected. Consequently, a second 5 ml. of blood was given to each calf from carrier 69597. Anaplasmosis then developed from 59 to 100 days after the first inoculation. These prolonged incubation periods, due to eperythrozoonosis, show that the recrudescence referred to above caused the eperythrozoonosis to become dominant to the anaplasmosis in cow 37177.

Calf 69912 was given 200 ml. of blood from an anaplasmosis-eperythrozoonosis nonsplenectomized carrier, 69596, and developed acute eperythrozoonosis. Because of the prolonged incubation periods of the first 7 calves (table 2), calf 69912 was placed in a pasture, Jan. 21, 1954, after he had recovered from the eperythrozoonosis, to determine if the latent anaplasmosis infection would overcome the interference of the eperythrozoonsis infection. The animal maintained a normal red blood cell count and showed no clinical signs of anaplasmosis or eperythrozoonosis for 243 days. Then, to determine if the interference of eperythrozoonosis could be overcome, this animal was inoculated at 15-day intervals with anaplasmosis-infective blood. It sickened with peracute anaplasmosis after the third inoculation.

Research at the Louisiana Station since October, 1953, shows that whichever infection, *i.e.*, eperythrozoonosis or anaplasmosis, is dominant in the carrier animal will establish itself first in the susceptible splenectomized calf. Research further shows that when eperythrozoonosis is dominant and the calf survives an acute or peracute attack, anaplasmosis infection will be blocked out indefinitely unless given a second or third dose of anaplasmosis inoculum (table 2). However, when eperythrozoonosis is dominant and the calf has a mild attack, anaplasmosis will become latent until the former subsides; then, eperythrozoonosis will become latent and anaplasmosis will occur either in the acute or peracute form. If a calf survives the attack of anaplasmosis and when the percentage of erythrocytes containing Anaplasma bodies begins to decline, clinical signs and positive blood films of eperythrozoonosis will again appear. The erythrocytes containing the Anaplasma bodies may or may not completely disappear from the blood stream, depending on the severity of the attack of eperythrozoonosis. After several days, eperythrozoonosis will again become latent and anaplasmosis will again become dominant. In some calves, this pattern recurs repeatedly until the animal seems to develop immunity to both diseases.

In other calves, the two diseases may exist simultaneously. Similar observations have been made in anaplasmosis-eperythrozoonosis mixed infections in sheep.[25] In such cases, the maximum of red blood cells containing Anaplasma bodies is sometimes as low as 30 per cent, yet the calf is just as sick clinically and becomes just as anemic as does the calf with 80 per cent of the erythrocytes containing Anaplasma bodies.[26] Additional work at this station has shown that some calves whose marginal body content never exceeded 10 per cent developed the same severe syndrome as those with much higher percentages.

In still other splenectomized calves, blood films indicate that the etiological agents of the two diseases compete for supremacy for several days before one or the other does become dominant.

SUMMARY

An interference phenomenon exists between anaplasmosis and eperythrozoonosis infections in the splenectomized calf. This interference is reciprocal.

Whichever infection may be dominant in the carrier animal will establish itself first in the anaplasmosis-eperythrozoonosis susceptible splenectomized calf.

When acute eperythrozoonosis is established first and the calf survives, the anaplasmosis infection may be blocked out indefinitely, unless a second or third inoculation of anaplasmosis-infective blood is given.

When a mild form of eperythrozoonosis is established first, it is subsequently replaced by acute or peracute anaplasmosis.

When anaplasmosis is the initial infection and even though it is acute, it is replaced in the splenectomized calf by eperythrozoonosis.

References

[1] Henle, W., and Henle, G.: Interference of Inactive Virus with Propagation of Virus of Influenza. Science, *98*, (1943): 87–89.

[2] Henle, W., and Henle, G.: Interference Between Inactive Viruses and Active Viruses of Influenza. III. Cross-Interference Between Various Related and Unrelated Viruses. Am. J. M. Sci., *210*, (1945): 362–369.

[3] Golub, O. J., and Wagner, J. C.: Studies on the Interference Phenomenon with Certain Members of the

Psittacosis-Lymphogranuloma Group of Viruses. J. Immunol., *59*, (1948): 59–70.

[4] Bang, F. B.: Cell Blockade in Newcastle Disease of Chickens and Chicken Embryos. J. Exptl. Med., *89*, (1949): 141–154.

[5] Hoskins, M.: A Protective Action of Neurotropic Against Viscerotropic Yellow Fever Virus in *Macacus Rhesus*. Am. J. Trop. Med., *15*, (1935): 675–680.

[6] Findlay, G. M., and MacCallum, F. O.: An Interference Phenomenon in Relation to Yellow Fever and Other Viruses. J. Path. and Bact., *44*, (1937): 405–424.

[7] Dalldorf, G., and Whitney, E.: A Further Interference in Experimental Poliomyelitis. Science, *98*, (1943): 477–478.

[8] Duffy, C. E.: Interference Between St. Louis Encephalitis Virus and Equine Encephalomyelitis Virus (Western Type) in the Chick Embryo. Science, *99*, (1944): 517–518.

[9] Vilches, A., and Hirst, G. K.: Interference Between Neurotropic and Other Unrelated Viruses. J. Immunol., *57*, (1947): 125–140.

[10] Ginsberg, H. S., and Horsfall, F. L., Jr.: Interference Between Mumps Virus and Pneumonia Virus of Mice (PVM). Fate of Mumps Virus in the Mouse Lung. J. Immunol., *67*, (1951): 369–377.

[11] Dalldorf, G.: The Sparing Effect of Coxsackie Virus Infection on Experimental Poliomyelitis. J. Exptl. Med., *94*, (1951): 65–71.

[12] Hanson, L. E., White, F. H., and Alberts, J. O.: Interference Between Newcastle Disease and Infectious Bronchitis Viruses. Am. J. Vet. Res., *17*, (1956): 294–298.

[13] Ziegler, J. E., Jr., and Horsfall, F. L., Jr.: Interference Between the Influenza Viruses. I. The Effect of Active Virus upon the Multiplication of Influenza Viruses in the Chick Embryo. J. Exptl. Med., *79*, (1944): 361–377.

[14] Lennette, E. H., and Koprowski, H.: Interference Between Viruses in Tissue Culture. J. Exptl. Med., *83*, (1946): 195–219.

[15] Jungeblut, C. W.: Reverse Interference Between Simian and Murine Poliomyelitis Virus in Guinea Pigs. Proc. Soc. Exptl. Biol. and Med., *68*, (1948): 615–617.

[16] Ledinko, N., and Melnick, J. L.: Interference Between Poliomyelitis Viruses in Tissue Culture. J. Exptl. Med., *100*, (1954): 247–267.

[17] Sugg, J. Y., and Magill, T. P.: The Serial Passage of Mixtures of Different Strains of Influenza Virus in Embryonated Eggs and in Mice. J. Bact., *56*, (1948): 201–206.

[18] Kilbourne, E. D., Anderson, H. C., and Horsfall, F. L., Jr.: Concurrent Infection with Influenza A and B Viruses in a Single Epidemic of Influenza. J. Immunol., *67*, (1951): 547–558.

[19] Tyzzer, E. E.: "Interference" in Mixed Infections of Bartonella and Eperythrozoon in Mice. Am. J. Path., *17*, (1941): 141–153.

[20] Foote, L. E.: New Information on Anaplasmosis. North Am. Vet., *35*, (1954): 19–21.

[21] Foote, L. E., Farley, H., Pearson, C. C., and Kliewer, I. O.: Intravenous Use of Ethyl Alcohol and Various Drugs in Treatment of Acute Anaplasmosis. North Am. Vet., *30*, (1949): 497–503.

[22] Foote, L. E.: Unpublished data, 1954.

[23] Foote, L. E., Brock, W. E., and Gallaher, B.: Icteroanemia, Eperythrozoonosis, or Anaplasmosis-like Disease of Swine Proved to Be Caused by a Filtrable Virus. North Am. Vet., *32*, (1951): 17–23.

[24] Splitter, E. J.: Eperythrozoonosis in Swine—Filtration Studies. Am. J. Vet. Res., *13*, (1952): 290–297.

[25] Splitter, E. J., Twiehaus, M. J., and Castro, E. R.: Anaplasmosis in Sheep in the United States. J.A.V.M.A., *127*, (1955): 244–245.

[26] Miller, J. G., Levy, H. E., Torbert, B. J., and Oglesby, W. T.: A Method of Screening Drugs to Be Used in the Treatment of Anaplasmosis—Results of Testing with Aureomycin and Terramycin. Proc. Book, AVMA (1952): 160–167.

The Pathology of Virus Diarrhea of Cattle in Indiana

R. G. CARLSON, D.V.M., Ph.D.; W. R. PRITCHARD, D.V.M., Ph.D.; L. P. DOYLE, D.V.M., Ph.D.

Lafayette, Indiana

VIRUS DIARRHEA (VD), as it occurs in Indiana, is an acute or chronic, contagious, febrile disease of cattle characterized by erosion and inflammation of the mucous membranes of the digestive tract accompanied by diarrhea, depression, dehydration, respiratory signs, and leukopenia.

Olafson *et al.*[1] observed ulcerations involving the oral mucosa, pharynx, larynx, esophagus, abomasum, and cecum, and hemorrhages in the omasum, small intestine, cecum, subcutaneous tissues, epicardium, and vagina in natural and experimental cases of VD in New York. Baker *et al.*[2] observed only oral lesions in 3 of 21 experimental cases. Wheat *et al.*[3] observed lesions in field cases of VD in California that were similar to those described by Olafson. The clinical features and brief pathological findings of naturally occurring and experimental cases of VD-Indiana have been described by Pritchard *et al.*[4-8]

The animals used in this study included some with fatal field infections and others with both field and experimental infections slaughtered in the acute, chronic, or recovery phase of the disease.

MATERIALS AND METHODS

Calves for experimental use were obtained from herds having no history of this disease and were housed individually or in pairs in tight isolation units. The ages of the animals varied from 2 weeks to over 1 year. All experimental animals were observed for at least one week before exposure. Clinical observations, the results of hematological determinations, and body temperatures were recorded daily or more frequently when indicated.

The agent used in this study was recovered from a field outbreak of VD-Indiana in a herd of adult dairy cattle. Intravenous inoculation of 5 ml. of defibrinated blood from this source produced signs of the disease in susceptible calves. Defibrinated blood collected from such a calf on the third to sixth day following exposure was shown to contain the agent by subsequent calf inoculation and served as the source material for this study. This material was stored at −40 C. in sealed ampules or screw-cap glass vials. Blood, urine, and various tissues were repeatedly examined for an agent other than virus diarrhea by various direct and cultural means with negative results.

Material from 20 calves was included in this study. Animals were slaughtered from nine days to three months following experimental exposure. Gross pathological alterations were recorded, photographs were made of some representative gross tissue changes, and specimens of most tissues were preserved in 10 per cent formalin or in Bouin's fluid for histological examination. The tissues were embedded in paraffin and stained with hematoxylin and eosin or phloxine.

RESULTS

All of the experimental animals showed a response following inoculation. The most pronounced signs were produced in 4 animals which were 10 to 15 months of age, while animals from 6 to 10 months showed the next most severe signs and lesions.

Clinical Examination.—There was a 1.0 to 3.0 F. rise in temperature on postinoculation (PI) day 3 (table 1). This initial febrile response did not occur until PI days 4 and 5 in 3 animals and was not observed at all in 3 other calves. The temperature rise was accompanied by a decrease in the total leukocyte count by an average of 4,000 cells in 11 calves. In the remainder, similar decreases did not occur until PI days 7 to 9.

The most marked clinical signs were associated with a second and more pronounced febrile response which occurred on PI day 7 or 8. The average temperature during this stage was 105.5 F., with an

Submitted as journal paper 1019, Purdue University Agricultural Experiment Station, Lafayette, Ind.

From the Department of Veterinary Science, Purdue University, Lafayette, Ind. Dr. Doyle is professor emeritus.

Taken in part from a thesis, "Studies on Virus Diarrhea in Cattle," submitted (by Carlson) to the faculty of the Graduate School, Purdue University, in partial fulfillment of the requirements for the Ph.D. degree, June, 1956.

The authors express their appreciation to Dr. D. B. Taylor and Mrs. W. D. Reece, for their assistance in the laboratory phases of this study, and to Dr. G. M. Neher, for his help in the preparation of the photographs.

This study was supported in part by research grants from the Lederle Laboratories Division of the American Cyanamid Co., Pearl River, N.Y., and the Agricultural Research Service, Animal Disease and Parasite Research Branch, U.S. Department of Agriculture.

Presented before the Combined Sections on Poultry and Research, Ninety-Third Annual Meeting, American Veterinary Medical Association, San Antonio, Texas, Oct. 15–18, 1956.

TABLE 1—Data Representative of the Postexposure Temperature Reaction

	Experimental animal							
	1	2	3	4	5	6	7	8
FIRST PHASE								
1) Initial level	101.8*	102.0	101.6	101.5	101.6	101.4	101.5	101.2
2) Increased to	104.3	104.7	101.8	104.4	102.5	103.3	102.3	102.6
3) Days postexposure	3	3	—	3	3	3	5	4
4) Duration (hr.)	24	24	—	96	24	24	72	24
SECOND PHASE								
1) Base interim temperature	102.2	101.8	101.8	102.7	101.6	101.1	101.8	101.8
2) Increased to	105.4	106.5	105.7	106.1	105.2	104.4	104.4	105.9
3) Days postexposure	8	8	8	7	8	8	10	8
4) Duration (hr.)	24	24	24	48	24	192	48	72

* Degrees Fahrenheit.

observed range of 104.4 to 108.0 F. Four animals did not show a marked temperature rise until PI days 10 to 12. The febrile reaction subsided within 24 hours in a majority of the animals but, in 5, temperatures remained high for two to eight days. Total leukocyte levels tended to remain low during the period between temperature peaks.

During the febrile period, all but 5 of the animals were depressed. Three of these 3 calves were hyperesthetic during and shortly after this period. Respiration and pulse rates were increased approximately 50 per cent during the acute stage. Anorexia and retarded rumination, associated with a degree of tympany and a foul-smelling breath, were observed in all but 2 of the animals. A dry cough and a serous nasal discharge were observed in one half of the animals. The nasal discharge became mucoid in 1 animal. From three to eight days after inoculation, 7 calves showed signs of lameness.

Diarrhea appeared within three to five days after inoculation in 3 animals but in the remainder was associated with the second temperature rise. In 2 calves, diarrhea was evident for only 24 hours; in the remainder, it was present for two to 20 days, with an average of five days. Free blood and small blood clots were evident in the feces of 3 animals and increasing amounts of mucus were observed in all the animals. Oral erosions, that could be seen on clinical examination, appeared at the same time as the diarrhea in 10 animals.

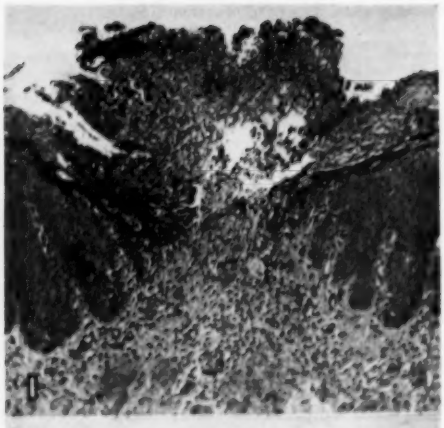
No change in the erythrocyte sedimentation rate was observed. A decrease in the hematocrit, hemoglobin, and total erythrocyte levels was evident (table 2) and reached a maximum by PI day 15 or 16 in 7 of 8 animals on which all of these determinations were made. The average decreases were 9.6 per cent for hematocrit levels, with a range of 5.5 to 17.0 per cent; 2.6 Gm. hemoglobin per 100 ml. of blood, with a range of 1.0 to 4.5 Gm.; and up to a 4.40 million decrease in total erythrocytes per cubic millimeter. These levels and also the total lekocyte counts tended to remain below pre-exposure levels for several weeks. Early changes observed in the experimental disease are shown (fig. 13).

In 3 animals, exacerbations and remis-

TABLE 2—Data Representative of Certain of the Postexposure Hematological Changes

	Experimental animal							
	1*	2*	3*	4*	5	6	7	8
TOTAL LEUKOCYTE LEVEL								
1) Initial level	7.0†	9.0	11.5	6.55	7.95	10.0	7.85	9.55
2) Depressed to	3.6	4.9	5.75	3.9	4.4	4.7	5.3	5.85
3) Days postexposure	3	3	8	7	3	4	8	9
4) Duration (hr.)	120	24	48	120	144	24	72	24
ERYTHROCYTE VOLUME								
1) Initial level	35.0‡	35.0	35.0	37.0	45.0	46.0	40.0	38.0
2) Depressed to	28.5	28.0	23.5	32.5	38.5	29.0	31.0	25.0
3) Days postexposure	6	6	8	10	8	10	15	16
HEMOGLOBIN (Gm./100 ml. of blood)								
1) Initial level	12.5	12.0	12.0	12.5	—	12.5	—	—
2) Depressed to	9.0	10.0	7.5	10.5	—	7.0	—	—
3) Days postexposure	6	6	10	10	—	8	—	—
AGRANULOCYTES								
1) Peak percentage	98	100	85	100	96	92	92	94
2) Days postexposure	4	3	8	3	2	12	9	11

* Indicates animal was necropsied in acute stage (9th to 13th day). † Leukocytes x 10^3 per cubic millimeter. ‡ Percentage of erythrocytes.



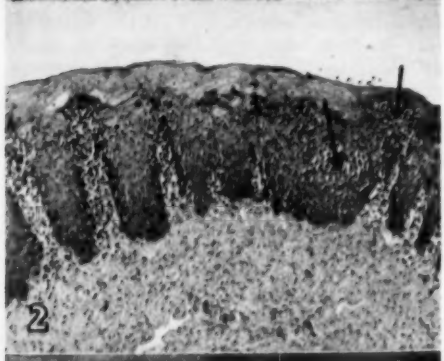


Fig. 1—A section of a lesion of the oral mucosa that extends through the basal cell layer. Appearance is typical of lesions of stratified squamous epithelial surfaces. The few infiltrating cells are polymorphonuclear leukocytes. Note the lack of other inflammatory signs. Experimental case. x100.

Fig. 2—Early stage of an esophageal lesion from an animal with an acute field infection. Note the cellular infiltration and edema of the papillae, the areas of necrosis (arrows), and the vacuolization of some of the more superficial cells. x 100.

Fig. 3—Gross esophageal lesion from an animal with an experimental infection, three months after exposure.

sions of clinical signs were noticed at approximately two-week intervals for two to three months before they were slaughtered. These animals were thin, had rough coats, showed frequent signs of bloat, and appeared stunted. They closely resembled cases of chronic VD observed under natural conditions.

TABLE 3—The Percentage of Occurrence of Certain Pathological Changes Observed in 20 Experimental Cases of Virus Diarrhea-Indiana

Lesion	Occurrence (%)
LOSS OF SURFACE EPITHELIUM	
Oral mucosa	50
Esophagus	50
Rumen and omasum	10
Vagina	5
LOSS OF SURFACE EPITHELIUM ASSOCIATED WITH CONGESTION, HEMORRHAGE, AND EDEMA	
Pharynx	30
Abomasum	90
Small intestine	90
Ileocecal valve	75
Cecum	10
Cecocolic junction	80
Colon	60
Rectum	80
Gallbladder	10
EDEMA	
Lymphatic tissue	90
Larynx and trachea	10
LYMPHOID EXHAUSTION	60

Postmortem Examination.—Affected animals showed evidence of dehydration, weight loss, diarrhea, and had rough coats. Gross and microscopic changes were most prevalent in animals with subacute, chronic, and recovering cases and were least prominent in those with early field and experimental cases. Certain of the changes observed in the experimental animals are tabulated (table 3).

Digestive System.—The agent appeared to have an affinity for the epithelium of the digestive tract, in particular, and there produced the most characteristic changes. Signs of inflammation and loss of surface epithelium were found from the muzzle to the anus. Most early lesions were not accompanied by a pronounced inflammatory cell reaction.

Lesions of the stratified squamous epithelial surfaces of the upper portion of the digestive tract were all of the same general nature (fig. 1 to 3). The superficial layers of such epithelial surfaces were most frequently affected but, similar to rinderpest,[9] initial involvement of cells immediately above the germinal layer did occur. The cells in affected areas had pyknotic and fragmented nuclei and homogeneously pink-stained or vacuolated cytoplasm. The

depth of these lesions varied. Some, especially those of the oral cavity, extended through the basal cell layer. A sharp line of demarcation usually existed between normal and affected areas.

Lesions on the muzzle and in the oral cavity first appeared as areas of hyperemia. Later local necrosis and sloughing occurred, leaving well-defined areas of erosion with raw-appearing bases. Lesions of the muzzle and lips were generally larger than those of the oral cavity. Oral lesions seldom exceeded 1.0 cm. in diameter in acute cases.

Edema, particularly of the lymphatic tissue, congestion, and slight hemorrhage were observed in the pharynx of 30 per cent of the experimental animals. Microscopic examination revealed degeneration and slight loss of the surface epithelium. Grossly visible pharyngeal erosions were uncommon in either animals with field or experimental infections.

Erosions of the esophagus were found in those cases showing oral involvement. The entire length of the esophagus was involved, with a few to numerous linear erosions (fig. 3). Early lesions appeared as well-defined, eroded areas with only slightly congested bases. Histologically, lesions of the esophagus had the same appearance as oral erosions although fewer extended through the basal cell layer. Pronounced inflammatory cell reactions were not observed in esophageal lesions.

The rumen, reticulum, and omasum were infrequent sites of gross involvement. Erosions and necrotic foci occurred but were few in number and were confined principally to the rumen pillars and omasal leaves.

Variable degrees of congestion, hemorrhage, and edema were observed grossly in the abomasums of all animals with acute and chronic cases (fig. 4 and 5). The pylorie portion was the most frequent site for severe involvement. Here, grossly visible erosions were evident in 40 per cent of the experimental animals. In the more chronic cases, these lesions varied from newly formed to completely healed. The lesions may involve only the more superficial tissue or extend entirely through the mucosa. Scarring was common in chronic and recovered cases.

Histological examination of the abomasum revealed considerable loss of surface epithelium. Edema of the mucosa may be marked and is associated with atrophy and dissociation of the cells lining the crypts. There was considerable alteration of the

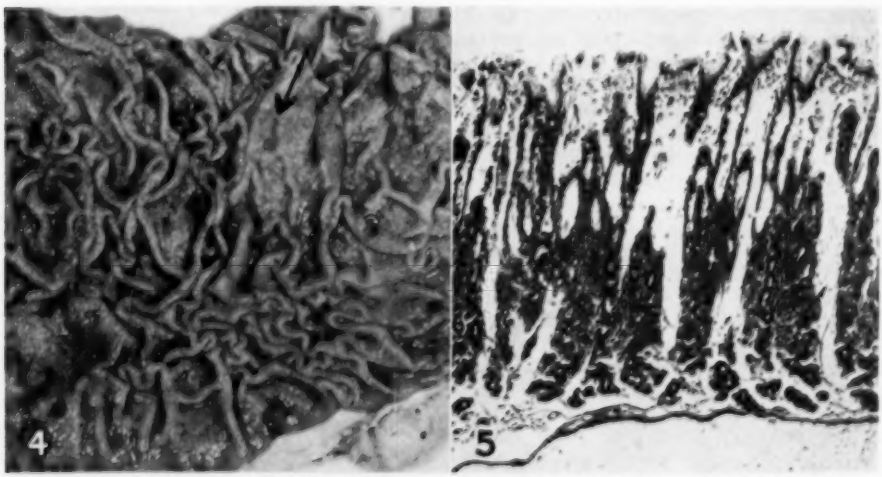


Fig. 4—Pyloric portion of the abomasum from an animal with a chronic experimental infection. Note the congestion and hemorrhage, the healed erosion (arrow), and the edema of the mucosa and submucosa as indicated by the thickened rugae.

Fig. 5—The histological appearance of the mucosa of the fundic portion of the abomasum from an experimental animal nine days after exposure. Note the edema of the mucosa and submucosa and the beginning desquamation of the surface epithelium. x 100.

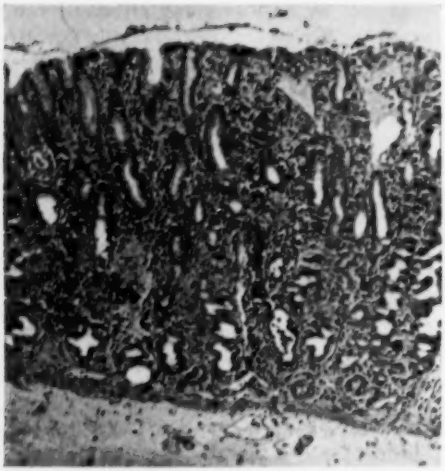


Fig. 6—The microscopic appearance of the pyloric portion of the abomasum from an animal with a chronic field case, showing metaplasia of the surface epithelium, disorganization, and atrophy of the pyloric glands 30 days after the onset of signs. x 100.

normal architecture in chronic cases (fig. 6).

A catarrhal enteritis, especially in the duodenum, was present in almost all animals and was associated with congestion, hemorrhage, and edema of the mucosa. Necrosis of the tips of the villi (fig. 7), apparently following loss of the surface epithelium, was common. Peyer's patches were frequently clearly evident through the serosal surface as a result of edema. Marked subserosal edema at the mesenteric attachment was found in 3 field cases.

Histological examination of the small intestine revealed a pronounced dilatation of the lymph spaces and edema between the degenerative and desquamating surface epithelium and the stroma of the villi (fig. 8 and 9). The loss of surface epithelium was pronounced in a majority of the animals examined. In animals with field infections which survived a severe attack, there was frequently a definite decrease in the length and number of villi remaining.

Changes very similar to those seen in the small intestine were observed in the cecum, colon, and rectum (fig. 10). The ileocecal valve, cecocolic junction, lower colon, and rectum were the areas most frequently involved. Petechiae on the longitudinal folds of the colon often formed what appeared to be continuous streaks of hemorrhage. Small blood clots and free blood were found in the lower colon and rectum of 3 of the more severely affected experimental animals.

The liver and gallbladder were not consistently involved. Hepatitis and cholangitis were observed in 2 experimental calves. Fatty degeneration was observed in most calves which were past the acute stage. Erosion, hemorrhage, and edema of the mucosa of the gallbladder occurred in 3 animals. Degeneration and necrosis of the cells of the mucosa and the associated mucous and serous glands (fig. 11) was evident in these calves. With the exception of degenerative changes in the epithelium of the pancreatic ducts, no specific lesions were observed in other organs of the digestive system.

Lymphatic and Blood Vascular Systems.—Palpable lymph nodes were grossly enlarged in 50 per cent, and Peyer's patches and lymph nodes of the abdominal viscera were enlarged and edematous in 90 per cent, of the acutely affected animals. Hemorrhage and congestion of the lymph nodes of the abdominal viscera were found occasionally in animals with field infections. Histological examination revealed lymphoid exhaustion in addition to edema in 60 per cent of the acutely affected experimental animals. In some instances, almost all of the lymphoid follicles exam-

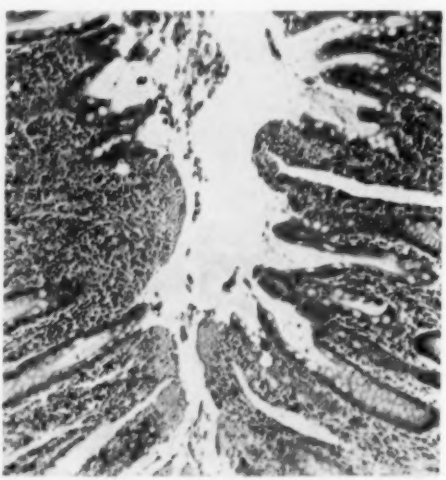


Fig. 7—Loss of surface epithelium with some necrosis of the tips of the duodenal villi in a recovering field animal. The infiltrating cells are principally lymphocytes. x 100.

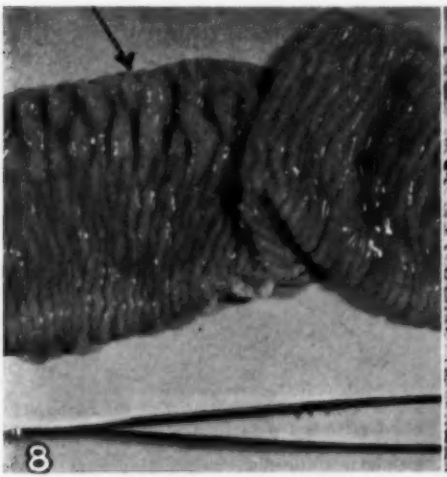


Fig. 8—Small intestine, showing edema of the mucosa and Peyer's patch (arrow).

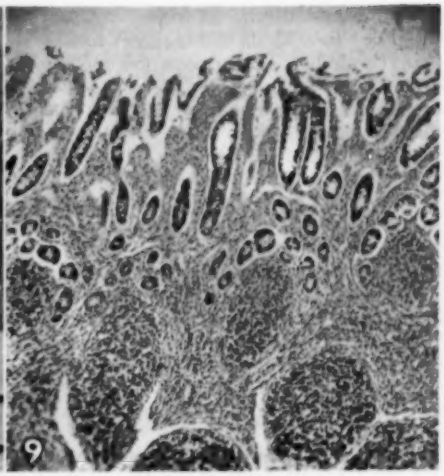


Fig. 9—The histological appearance of the small intestine in an animal with an acute experimental infection, showing loss of surface epithelium, degeneration, and atrophy of epithelial cells in the crypts, and edema of the mucosa and Peyer's patch. x 40.

ined appeared depleted, while in others only a few were affected.

The gross appearance of the spleen was normal in experimental calves. A depletion of the germinal centers in acute cases was the only histological change observed. The necrotic foci, which were occasionally observed in both the spleen and lymph nodes, were associated with but did not appear to involve an entire germinal center. No pathological alteration of the hematopoietic system was found.

Subendocardial hemorrhages at the base of the papillary muscles in the left ventricle, subepicardial hemorrhages near the base of the heart, and atony of the myocardium were the principal cardiac lesions observed. Serous infiltration of the fat at the base of the heart was observed in long-standing cases.

Respiratory System.—Gross or microscopic lesions of the mucosa, resembling those of the alimentary tract, were not observed. Acute, fatal field cases frequently showed congestion of the nasal cavity, larynx, and trachea, and a tenacious mucous exudate. Edema, around the larynx and trachea, was noted in these field cases and in 2 of the experimental calves. Patchy congestion of the nasal mucosa and trachea was observed in acute experimental animals but no exudate was present.

In the lungs, minor areas of consolidation, atelectasis, and both alveolar and interlobular emphysema were common. These changes appeared to be associated with the general condition of the animal rather than being specific changes brought about by the causative agent.

Urogenital System.—The kidneys commonly showed congestion of the cortex

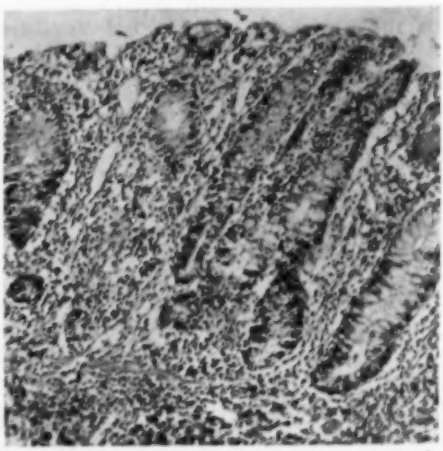


Fig. 10—Microscopic appearance of the mucosa in the cecocolic region, showing complete loss of surface epithelium, mucoid degeneration of the epithelium of the crypts, and edema of the stroma. x 100.

and corticomedullary junction. Observed changes in the tubule cells seldom consisted of anything but albuminous degeneration. Serous infiltration of the perirenal fat occurred in 2 chronic experimental cases. Animals slaughtered in the chronic or recovery stage showed atrophic changes primarily in the collecting tubules.

Small areas of degeneration and necrosis of the urinary bladder epithelium were observed in 2 field cases and were associated with congestion of the bladder wall at those points. In 1 experimental calf, the vaginal mucosa was eroded.

Nervous System.—Gross involvement of the central nervous system was not observed. Slight perivascular cuffing and gliosis occurred in 2 field and 1 experimental cases. No inclusion bodies were found in any tissue.

Endocrine, Skeletal Musculature, Integumentary, and Skeletal Systems.—No specific pathological alterations were observed in any of these systems. The only lesions observed were those commonly associated with debilitating conditions, e.g., scurvy, dehydrated skin, and atrophy of many of the parenchymatous organs.

DISCUSSION

The pathological changes observed in this disease involved primarily the mucosa of the alimentary tract and lymphatic tissue. These changes were observed to be present in experimental animals as early as the ninth day and as late as three

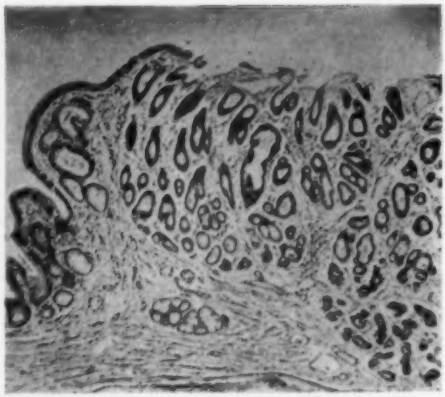


Fig. 11—An acute lesion of the gallbladder. There is extensive loss of surface epithelium, dilatation of the glands of the mucosa, and atrophy of the glandular epithelium. Edema is present but only slight cellular infiltration. x 40.

months after exposure. There was a close resemblance between the lesions observed in naturally occurring and experimental cases of the disease. The changes were, in general, more severe in field cases than in experimentally induced cases. Under field conditions, the mortality rate varied from 0 to 50 per cent and averaged less than 10 per cent. No deaths occurred in this series of experimentally infected animals.

Necrosis and desquamation of cells from the surfaces of mucous membranes was characteristic. The mucosa of the alimentary canal was most frequently affected,

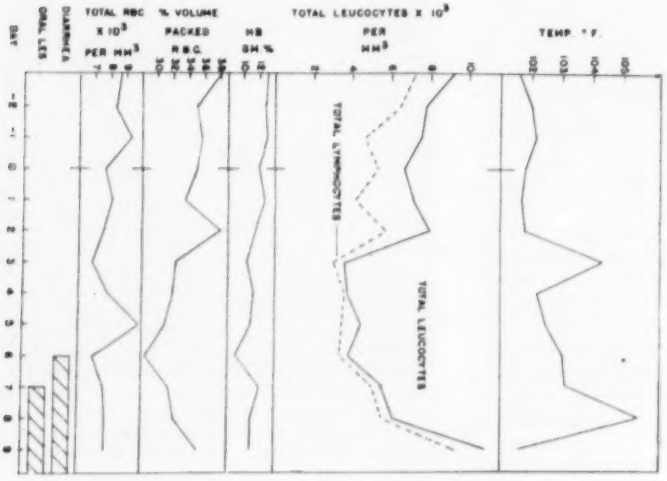


Fig. 12—The clinical findings in a calf following experimental infection. Necropsy on the tenth day revealed pronounced acute lesions of virus diarrhea.

but similar lesions did occur on other mucosal surfaces. Congestion and hemorrhage appeared to be secondary to the loss of surface epithelium and occurred more frequently where unstratified cell layers were present. The lack of an inflammatory cell reaction to these changes was obvious in many cases. Abomasal ulcerations, particularly in the region of the pylorus, and hemorrhage and congestion of the ileocecal valve are frequently found in otherwise normal animals. Care should be taken to avoid erroneous interpretation of these lesions.

Edema appears to play an important role in causing certain of the signs and lesions of VD-Indiana. In acute cases, many of the pathological changes that occur from the abomasum to the anus seem to be related to the degree of edema present in the mucosa and submucosa. This relationship was most obvious in the small intestine where necrosis and desquamation of the epithelium were consistently associated with edema of the mucosa. Edema may also cause the diarrhea and the rapid weight loss and dehydration observed in the disease. Lymphoid tissue, particularly that of the intestinal wall but also that in other areas, is frequently edematous in the acute disease. This results in a high percentage of the animals having clinically detectable enlargement of the external lymph nodes.

On the basis of the report by Olafson *et al.*[1] there are no apparent major differences in the gross pathology of VD-New York and VD-Indiana. Experimental evidence indicates that VD-Indiana may be caused by a different "type" of the same virus that causes VD-New York rather than being a distinctly different disease.[7]

The tissues from 10 natural cases of mucosal disease[10,11] were examined in conjunction with this study in an effort to compare the pathological changes in these two conditions. In mucosal disease, they are much more severe than in VD, even though the distribution and character of many of the lesions are similar. In mucosal disease, erosions, ulcerations, and destruction of lymphatic tissue are widespread in the alimentary tract. Involvement of the oral cavity, esophagus, rumen, and omasum may be extensive. In mucosal disease, atrophic and metaplastic changes, erosions, ulcerations, hemorrhage, congestion, edema, and associated changes are very conspicuous from the abomasum to the anus on both gross and microscopic examination.

Mucoid degeneration of the epithelial cells of the mucosa, particularly of the intestinal diverticula, is frequently observed from the duodenum to the rectum in mucosal disease. In the cecocolic region, these changes closely resemble rinderpest.[9] An additional resemblance to rinderpest is seen in the lymphatic tissue of the small intestine. In both conditions, little lymphatic tissue may remain. Peyer's patches become degenerated, necrotic, and frequently slough. In mucosal disease, such lesions often show remarkably little cellular reaction and rapidly become covered with metaplastic epithelial cells. Lesions of this nature have not been observed in VD-Indiana.

In addition to the pronounced lesions of the mucosa and lymphatic tissue of the alimentary tract in mucosal disease, changes are frequently observed in other organs. Focal necrosis of the liver with an associated lymphocytic infiltration commonly occurs in mucosal disease. Atrophic and degenerative changes in the liver and kidneys are much more pronounced than in VD-Indiana. Focal necrosis of the germinal centers in the spleen and lymph nodes are also observed in VD-Indiana but not to as great an extent as in mucosal disease.

SUMMARY

An effort was made to characterize the pathology of virus diarrhea (VD) of cattle as it occurs in Indiana. The information was determined from clinical examination and necropsy of animals with fatal field infections and field and experimental animals slaughtered in the acute, chronic, or recovery phase of the disease.

There was considerable variation in the degree of pathological involvement observed in both experimental and field cases of VD-Indiana. The changes were, in general, more severe in field cases than in the experimental disease. There was, however, a close resemblance between the character of the lesions observed.

The mucosa of the alimentary tract, from the muzzle to the anus, and lymphoid tissue were the most nearly constant sites for pathological alteration. Lesions of the digestive tract were primarily of an erosive and ulcerative nature and were associated with edema, congestion, and hemor-

rhage, particularly in those areas where only a single cell layer separates the lumen of the tract from the underlying vascular stroma. Edema and exhaustion of the germinal centers were the principal changes observed in lymphatic tissue. The majority of changes involving other tissues were considered to be secondary and nonspecific.

References

[1] Olafson, P., MacCallum, A. D., and Fox, F. H.: An Apparently New Transmissible Disease of Cattle. Cornell Vet., *36*, (1946): 205–213.

[2] Baker, J. A., York, C. J., Gillespie, J. H., and Mitchell, G. B.: Virus Diarrhea in Cattle. Am. J. Vet. Res., *15* (1945): 525–531.

[3] Wheat, J. D., McKercher, D. G., and York, C. J.: Virus Diarrhea in California. Calif. Vet., *7*, (1954): 26–27, 35.

[4] Pritchard, W. R., Bunnell, D., Moses, H. E., and Doyle, L. P.: Annual Report. Purdue Agric. Exper. Sta., 1954.

[5] Pritchard, W. R.: A Transmissible Erosive Gastro-Enteritis of Cattle. Iowa State Coll. Vet., *17*, (1954–1955): 155–158.

[6] Pritchard, W. R.: The Mucosal Diseases of Cattle—Epizootiology, Symptomatology, and Experimental Studies. Proc. Book, AVMA (1955): 37–42.

[7] Pritchard, W. R., Carlson, R. G., Moses, H. E., and Taylor, D. B.: Virus Diarrhea and Mucosal Disease. Proc. U.S. Livestock San. A. (1955): 173–188.

[8] Pritchard, W. R., Taylor, D. B., Moses, H. E., and Doyle, L. P.: A Transmissible Disease Affecting the Mucosae of Cattle. J.A.V.M.A., *128*, (1956): 1–4.

[9] Maurer, F. D., Jones, T. C., Easterday, B., and DeTray, D.: The Pathology of Rinderpest. Proc. Book, AVMA (1955): 201–211.

[10] Ramsey, F. K., and Chivers, W. H.: Mucosal Disease of Cattle. North Am. Vet., *34*, (1953): 629–633.

[11] Ramsey, F. K.: The Pathology of a Mucosal Disease of Cattle. Proc. Book, AVMA (1954): 162–166.

The Endogenous Development of *Eimeria Zurnii*, a Pathogenic Coccidium of Cattle

LEONARD REID DAVIS, Ph.D., and GEORGE W. BOWMAN

Auburn, Alabama

EIMERIA ZURNII (Rivolta,[12] 1878), Martin,[10] 1909, one of the ten species of coccidia occurring in cattle in the southeastern United States, is probably the most pathogenic, as it, along with *Eimeria bovis*, is most frequently involved in clinical coccidiosis.[6] In 1878, Pröger,[11] a veterinarian in Switzerland, observed that calves died following a severe enteritis, and he submitted pieces of intestine to F. A. Zürn for examination. Zürn [15] published a description of the numerous parasites that he found in the epithelial cells and contents of the intestine. Zschokke [16] and Hess [9] confirmed the cause of *rote Ruhr*, or "red diarrhea," and included drawings of the distended, parasitized glands of the intestine.

Various descriptions have been made of the stages in the intestine, but these have been based on material obtained postmortem from naturally infected, fatal, or clinical cases, some of which have been mixed infections. One of the most complete descriptions is the publication by Smith and Graybill.[14] The present paper supplies information on the endogenous development in calves artificially inoculated with pure cultures of sporulated oocysts of *E. zurnii*.

MATERIALS AND METHODS

Fourteen calves ranging in age from 2 days to 8 weeks were used in these experiments. Methods of raising calves experimentally at this laboratory have been described.[5, 6] No muzzles were used on the calves in the present study. The oocysts were obtained from feces of both naturally and artificially infected calves showing oocysts of *E. zurnii* uncontaminated by other species. Sporulation was accomplished by the method of Hammond and Davis.[7] The number of oocysts administered ranged between 500,000 and 50,000,000. Calves were killed at intervals between two and 20 days after inoculation. None were killed after that, because it was reported [5] that in 117 inoculations with *E. zurnii*, which resulted in 94 "takes," more calves showed the greatest numbers of oocysts discharged on the nineteenth and twentieth days than at any other time. Unpublished data from the same experiments showed that in a group of 40 calves with high peaks of oocyst discharge, the prepatent period ranged between eight and 28 days (average, 14.0 days), the highest peaks occurred between eight and 41 days (average, 19.5 days), and the patent period ranged from three to 28 days (average, 12.8 days).

Oocysts suspended in water were given orally by means of a nippled flask in single inoculations, with the exception of the following: 1 calf was inoculated on the second and ninth days before killing, another on the third and tenth days, and one on the ninth and tenth days. The remainder were killed on the following days of the cycle: 5, 6, 7, 7, 12, 13, 14, 15, 16, 17, and 19 days. All calves were examined immediately after they were killed.

Smears in saline were examined microscopically, and tissues for sections were obtained approximately every 6 feet throughout the intestines. Fixation was accomplished regularly in Zenker's and, occasionally, in Bouin's fluid or formalin. Hematoxylin and eosin or Giemsa stains were used.

RESULTS AND DISCUSSION

Asexual Stages.—Schizonts in Tissue Sections (fig. 1 to 6).—On the second day, the youngest and smallest parasites observed were mostly uninucleated; apparently they were sporozoites. They had penetrated the tissues of the small intestine approximately 12 and 18 feet below the abomasum. One, only 3.5 μ in diameter, had penetrated the glands and was lodged next to the muscularis mucosae; another one was found between the nucleus of a cell and the lumen of the gland. These young schizonts had a clear area surrounding the centrally located karyosome in the nucleus. As in most of the younger stages, the parasites invariably were encircled by a clear zone separating them from the host cytoplasm (fig. 2), giving the appearance of shrinkage due to fixation and other manipulations before sectioning.

By the third day, the schizonts were rounded, mostly uninucleate bodies averaging 7.0 μ in diameter (fig. 2). The

From the Regional Animal Disease Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Auburn, Ala.

karyosome was centrally located in a non-staining clear area. The schizonts were found in the upper small intestine 18 to 24 feet below the pylorus. No stages were found on the fifth day.

On the sixth day, developing schizonts with many nuclei (fig. 3) were found in the small intestine 8 feet below the pylorus and at 16 feet above the cecum. They were distally located (between the host nucleus and the apex of the cell). They ranged between 3.5 by 4.9 μ and 7.0 by 9.8 μ, the latter dimensions being those of a nearly mature schizont, with merozoites beginning to elongate. No parasites were found in the sections made on the seventh day.

In the calf inoculated nine and ten days prior to slaughter, the only parasite observed was in the distal tip of a cell in the upper cecum. It was a body 1.4 to 2.8 μ in size. This was probably a merozoite that had rounded up and was beginning to form either another schizont or a gametocyte.

Developing schizonts at 12 days were found in the cecum and lower colon, ranging in size between 4.2 and 7.0 μ. They were distally located in the cells. At 14 days, many more stages were found than in the calves killed earlier. Most were found in the areas about 18 and 32 feet above the ileocecal valve. Early schizonts with numerous nuclei measured 5.6 to 7.0 μ in size, while mature ones containing merozoites and residual bodies were 5.6 by 11.9 μ in elongated ones and 9.4 by 11.2 μ in the more rounded ones. They were found on either side of the nucleus. The merozoites in the schizonts measured 1.2 by 6.3 μ in the sections.

Schizonts on the fifteenth day contained between 24 and 36 merozoites (fig. 4 and 5) and measured 8.4 by 11.9 μ to 10.5 by 16.1 μ, averaging 9.5 by 12.3 μ. Some schizonts were partially extruded into the lumens of the intestinal glands. Most were located in the small intestine at 1 and 18 feet above the ileocecal valve. Some of these were immature schizonts with only about five to eight nuclei showing. One 3.5 μ-sized parasite was found in the apex of a cell, and it appeared to be a rounded-up merozoite, with an eccentrically located karyosome. One schizont was found within 6 feet of the pylorus, and its location was probably due to its being delayed some place in the digestive tract, such as the rumen.

Roudabush [13] reported that some sporozoites of *Eimeria separata* in rats were found free in the lumen of the cecum at three days. Hammond *et al.*[8] reported that surgical stenosis just prior to inoculation with oocysts of *E. bovis* resulted in the greatest concentration of schizonts in the region from about 3 feet anterior to the partial blockage to about 2 inches below it.

The present authors occasionally have found sporulated oocysts, apparently from the inoculum, being passed in the feces two days after inoculation, and also found that closed cellophane tubes containing sporulated oocysts and protected by perforated gelatin capsules or by perforated aluminum capsules were intact in the rumen of calves as long as two and three days, respectively, after inoculation. Any delay in the passage of oocysts through the long digestive tract of a calf could possibly enable the sporozoites to be excysted and ready to penetrate the intestinal wall near the stomach instead of the usual site farther down. Also, a reverse peristalsis of the intestine may account for a schizont in the anterior portion of the intestine.

The calves examined on the sixteenth

Legends for Illustrations on Opposite Page

Figures 1 to 16

Fig. 1 to 3—Early, dividing schizonts.

Fig. 4—Mature schizont with merozoites sectioned transversely.

Fig. 5 to 6—Mature schizonts with merozoites sectioned longitudinally through nuclei.

Fig. 7—Merozoite in cytoplasm of a cell.

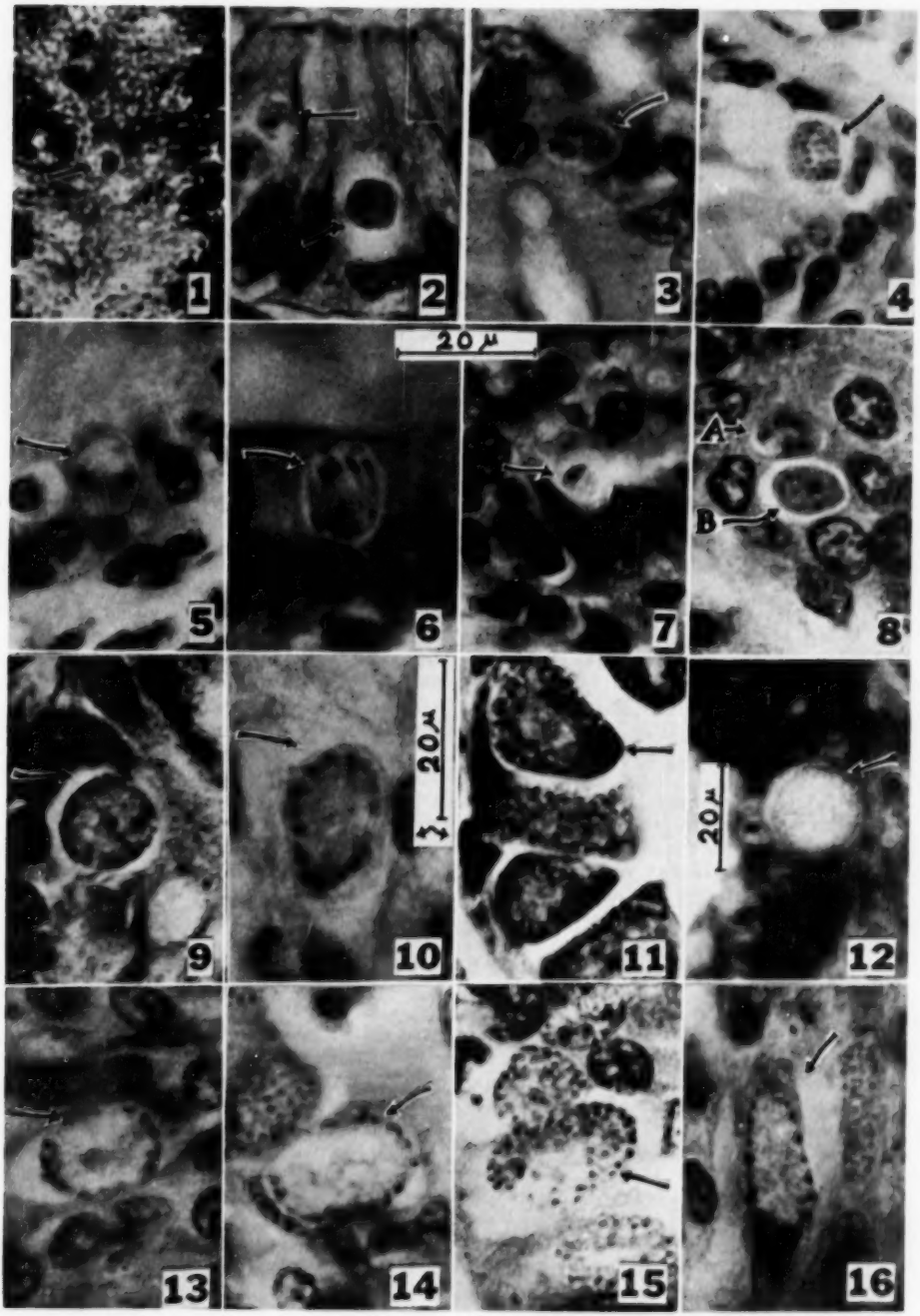
Fig. 8—Multinucleate, early microgametocyte (A) and uninucleate, early macrogametocyte (B).

Fig. 9 to 11—Developing macrogametes, showing peripherally located plastic granules.

Fig. 12—Mature oocyst with "shell."

Fig 13 to 15—Developing microgametocytes.

Fig. 16—Mature microgametes arranged peripherally in a microgametocyte.



—*Photographed by Leonard Reid Davis*

Unretouched photomicrographs of tissue sections showing stages in the life cycle of Eimeria zurnii from experimentally infected calves. The upper (horizontal) 20-μ scale applies to all photomicrographs, with the exception of figures 10 to 12, which have their own scales (vertical).

and seventeenth days were negative, but the calf killed on the nineteenth day revealed many more parasites than at any other time. Schizonts ranged between 7.0 by 10.5 μ and 10.5 by 16.8 μ, with an average of 9.6 by 13.2 μ. Some schizonts had residual bodies averaging 2.1 μ in diameter.

Merozoites (Fig. 4 to 7).—In fresh smears in saline, merozoites were first found at seven days 1 foot above the ileocecal valve and in the rectum. They averaged 11.2 μ in length by 1.7 μ in width. At ten days, they were found in the small intestine beginning 12 feet from the abomasum and extending to within 6 feet of the ileocecal valve. The greatest number was 9 per x 440 field at midintestine, 24 feet below the abomasum. They ranged in length between 5.4 and 10.8 μ, with an average of 7.5 μ. The variation in length may have been an indication of more than one generation of merozoites.

At 14 days, merozoites were found in the lower 6 feet of the small intestine. They ranged between 7.0 and 12.0 μ in size. At 15 days, they were found beginning at 2 feet from the abomasum to a point 12 feet above the ileocecal valve; they ranged between 10.0 and 12.0 μ in size.

At 19 days, merozoites were located beginning 12 feet above the ileocecal valve and extending into the cecum, colon, and rectum, with the greatest concentration at 6 feet above the lower end of the small intestine. Four to five were observed in each x 440 field on fresh smears. They ranged in size between 8.1 and 12.2 μ, with an average of 10.4 μ.

Stained merozoites had blue cytoplasm with two lightly stained pink refractile globules, with a lightly stained nucleus toward the tapering end. After penetrating the cytoplasm of a cell, the merozoites had a stocky appearance (fig. 7), as contrasted with the merozoites still in the intact schizont (fig. 5 and 6).

The slightly larger average size of the mature schizonts late in the cycle and the predominantly distal location of the ones early in the cycle were the only indicants of more than one asexual generation. In order to produce the great number of gametes and oocysts later in the cycle, and considering the small size of the schizonts and the relatively small number of merozoites in each, it is possible that more than one generation is developed, as in Eimeria in other hosts. In cattle, *E. bovis* was found to have only one asexual generation but the schizonts were distinguished by being macroscopic; 20 of them were examined by dilution counting with a hemocytometer, resulting in an estimated average number of 120,000 merozoites each.[5]

Sexual Stages.—Macrogametocytes (Fig. 8, B, and 9 to 11).—A young macrogametocyte was found in the cecum at 12 days. It measured 7.0 μ and had a 1.4-μ karyosome. At 14 days, the macrogametocytes were sparse and were located in the small intestine 18 feet above the ileocecal valve. Each was spherical, measuring between 4.2 and 5.6 μ, and had eccentrically located karyosomes. At 15 days, macrogametocytes ranged between 8.4 by 10.5 μ and 14.0 by 14.7 μ, with an average of 11.2 by 13.4 μ. Most were in the middle and lower colon, but two were found in the small intestine within 3 feet of the abomasum.

At 19 days, macrogametocytes were more numerous than before, but were only in the lower colon and rectum. It was common to find cross sections of glands with a macrogamete in every epithelial cell (fig. 11), and some were found in surface epithelial cells. They ranged in size between 7.0 by 9.4 μ and 12.6 by 14.0 μ, with an average size of 10.6 by 13.5 μ.

The macrogametocytes had a distinctive central nucleus and an irregular karyosome (fig. 9 and 10). The peripherally arranged plastic granules (fig. 10), sometimes in a double row, resembled those of *E. bovis*,[3] another coccidium in cattle.

Microgametocytes (Fig. 8, A, and 13 to 16).—Microgametocytes were found at 15 and 19 days. They were distinguishable from macrogametocytes by the absence of a centrally located nucleus and by having numerous basophilic bodies (fig. 13 and 15) and, as maturation occurred, a single layer of peripherally located, densely stained, comma-shaped microgametes (fig. 16).

At 15 days, the microgametocytes were 9.0 by 12.3 μ to 10.0 by 13.0 μ in size. At 19 days, they ranged between 7.0 by 8.4 μ and 15.0 by 20.0 μ, with an average size of 9.7 by 13.8 μ. The elongated shape of some appeared to be due to the pressure of adjacent cells. Most of the microgametocytes were located in the lower colon and rectum, but some were also

found in the midcolon. They usually occurred in greater numbers near the lumen of the digestive tract, mostly intermingled among macrogametocytes but in fewer numbers.

Oocysts (Fig. 12).—Immature oocysts were found with incompletely developed "shells" in fresh smears of the cecum at 12, 13, and 14 days. At 14 days, ten immature oocysts were also found in fresh smears about 2 feet above the ileocecal valve. Oocysts were found in the cecum and colon at 19 days; some were still immature, while others had distinct "shells" and appeared ready to be discharged. When present in the oocysts with definite shells, the nucleus was smaller and more compact than in the younger oocysts. Some oocysts that looked more mature than others had no nucleus, and the protoplasm was diffused and homogeneous in appearance (fig. 12). Oocysts with shells ranged between 12.2 and 25.7 μ long (average, 17.6 μ) by 12.2 to 12.6 μ wide (average, 14.3 μ), being very near the size that Christensen [1] reported for expelled oocysts of *E. zurnii* in other Alabama cattle.

The above descriptions of the endogenous stages are similar to the ones found at necropsy by Smith and Graybill.[14] Even though they knew that they were seeing more than one species, as based on size and morphology, they believed that the endogenous stages which they found in the clinical cases were due to the small, elliptical species (probably *E. zurnii*), because of the greater prevalence of these oocysts in sections of the tissues as compared with the large species, which was rarely found. Of the numerous reports of necropsies of animals with clinical cases, the one by Smith and Graybill [14] is probably the most complete from the standpoint of histopathology. The present authors have seen similar damage in 10 naturally acquired fatal cases involving *E. zurnii* in cattle. The sloughing of the epithelium and destruction of areas of tubules followed by necrosis are somewhat different from the damage by *E. bovis*. In cases involving *E. bovis*, the endothelial cells of the endothelial lining of the lacteals are also invaded, causing the eventual distention and possible rupturing of the tips of the villi by the growing, macroscopic schizonts.[5] It is also different from the damage produced by *Eimeria alabamensis* in cattle,[4] in which the site of the infection was found to be the nucleus of the host cell, with an eventual destruction of the nucleus by one or more sexual or asexual stages. Even though *E. alabamensis* was shown to be fairly nonpathogenic under field conditions or when small numbers of oocysts were inoculated, it produced signs of disease and even death when large numbers were given.[3]

SUMMARY

1) Sporulated oocysts of *Eimeria zurnii* (Rivolta, 1878), Martin, 1909, one of the most pathogenic of the coccidia of cattle, were administered to 14 young calves that were killed at various periods between two and 20 days after infection to study the endogenous stages.

2) In fresh smears and tissue sections, schizonts were first seen at two days and were still observed as late as 19 days after infection. They were found in the upper, middle, and lower portions of the small intestine and in the cecum and colon. Mature schizonts had between 24 and 36 merozoites and averaged 9.6 by 13.2 μ in size.

3) Merozoites, first found at seven days, were between 5.4 and 12.2 μ in size. They were located in the small intestine, cecum, colon, and rectum.

4) Macrogametocytes, averaging 10.6 by 13.5 μ, were identified at 12, 14, 15, and 19 days, mostly in the lower small intestine, cecum, colon, and rectum, with the exception of two found in the upper small intestine.

5) Microgametocytes were found only at 15 and 19 days, and averaged 9.7 by 13.8 μ at 19 days. The microgametes were small, densely stained, comma-shaped bodies, arranged peripherally in mature microgametocytes. Microgametocytes were fewer in number than macrogametocytes.

6) Immature oocysts were first apparent at 12 days. They were found in the cecum and colon, with fewer numbers located in the lower small intestine.

References

[1] Christensen, J. F.: The Oocysts of Coccidia from Domestic Cattle in Alabama (U.S.A.), with Descriptions of Two New Species. J. Parasitol., *27*, (1941): 203–220.

[2] Davis, L. R.: Portable Pens for Raising Healthy Dairy Calves. BAI, U.S.D.A., Washington, D. C., (Sept., 1949): 1–4 (mimeo).

[3] Davis, L. R., Boughton, D. C., and Bowman, G. W.: The Biology and Pathogenicity of *Eimeria Alabamensis*, Christensen, 1941, an Intranuclear Coccidium of Cattle. Am. J. Vet. Res., *16*, (1955): 274–281.

[4] Davis, L. R., Boughton, D. C., and Bowman, G. W.: The Endogenous Development of *Eimeria Alabamensis*,

Christensen, 1941, an Intranuclear Coccidium of Cattle. J. Protozool., suppl., *2*, (1955): 8.

[6] Davis, L. R., and Bowman, G. W.: Coccidiosis in Cattle. Proc. U. S. Livestock San. A. (1951): 39–50.

[7] Hammond, D. M., Davis, L. R., and Bowman, G. W.: Experimental Infections with *Eimeria Bovis* in Calves. Am. J. Vet. Res., *5*, (1944): 303–311.

[8] Hammond, D. M., and Davis, L. R.: An Improved Method for Sporulating Oocysts in Bovine Fecal Material. Am. J. Vet. Res., *5*, (1944): 70–71.

[9] Hammond, D. M., Bowman, G. W., Davis, L. R., and Simms, B. T.: The Endogenous Phase of the Life Cycle of *Eimeria Bovis*. J. Parasitol., *32*, (1946): 409–427.

[10] Hess, E.: Die rote Ruhr des Rindes (dysenteria haemorrhagica coccidiosa). Schweiz. Arch. f. Tierheilk., *34*, (1892): 105–128.

[11] Martin, A.: Les Coccidioses des animaux domestiques. Rev. Vét., Toulouse, *60*, n.s. *34*, (1909): 273–285.

[12] Pröger: Psorospermienkrankheit (Gregarinose) beim Kalbe. Ber. Veterinärw. Königr. Sachs., *22*, (1878): 113–115.

[13] Rivolta, S.: Della gregarinosi dei polli e dell' ordinamento delle gregarine e dei psorospermi degli animali domestici. Gior. Anat., Fisiol. e Patol. Animali, *10*, (1878): 220–235.

[14] Roudabush, R. L.: The Endogenous Phases of the Life Cycles of *Eimeria Nieschulzi*, *Eimeria Separata*, and *Eimeria Miyairii* Coccidian Parasites of the Rat. Iowa State Coll. J. Sci., *11*, (1937): 135–163.

[15] Smith, T., and Graybill, H. W.: Coccidiosis in Young Calves. J. Exptl. Med., *28*, (1918): 89–108.

[16] Zürn, F. A.: Die kugel- und-eiförmigen Psorospermien als Ursache von Krankheiten bei Hausthieren. Vorträge Thierärzte, *1*, (1878): 37–68.

[17] Zschokke, E.: Beobachtungen über die rote Ruhr. Schweiz. Arch. f. Tierheilk., *34*, (1892): 49–63.

Isolation of *Vibrio Fetus* from Bulls

W. N. PLASTRIDGE, Ph.D.; E. C. WALKER, B.S.; L. F. WILLIAMS, M.S.;
E. F. STULA, D.V.M.; E. M. KIGGINS, M.S.

Storrs, Connecticut

IT IS generally recognized that the bull is a primary factor in the transmission of *Vibrio fetus* infection in cattle and that serological methods are of little value for detecting bulls which carry *V. fetus*. Diagnostic techniques that involve the use of guinea pigs, or exposure of heifers by natural service or artificial insemination (AI), are not practical for routine use. Cultural methods appear to offer the best means of detecting *V. fetus* in bulls. However, the presence of saprophytic bacteria in aseptically drawn semen and in preputial washings makes isolation of *V. fetus* by primary culturing in fluid mediums difficult and often impossible. Solid mediums fail to support the growth of some strains of *V. fetus* under the usual method of incubation in an atmosphere of 10 per cent carbon dioxide and 90 per cent air.

The results obtained by use of an improved cultural procedure are presented here.

MATERIALS AND METHODS

Portions of 0.5 ml. of 544 samples obtained in the routine collection of semen from 149 bulls in four New England AI centers were submitted for examination. The interval between retests was one to two months. The samples were packed in an insulated container with a can of frozen water and were either delivered to the laboratory on the day collected or were shipped and received on the following day, with the exception of a few which were received 48 hours, or longer, after collection. Semen samples that were frozen for transportation were found to be unsatisfactory. Preputial washings were obtained from 19 bulls by infusing about 50.0 ml. of tryptose broth into the preputial sac, massaging the prepuce, and aspirating as much of the fluid as possible. The washings were refrigerated during transportation and cultured about three hours after collection.

A 4.0-mm. loopful of semen plus a loopful of thiol medium (Difco) were streaked over the surface of a fresh blood agar plate (5.0% defibrinated ox blood in nutrient agar prepared with desiccated meat infusion, Difco, 0.5% NaCl and adjusted to pH 7.0 ± 0.2). Three loopfuls of semen were mixed with 1.0 ml. of thiol medium (1:30 dilution) and 2 loopfuls of this dilution were streaked on a second blood agar plate. Preputial samples were cultured in a similar manner both before and after centrifugation at 1,000 g for 20 minutes.

At the start, the plates were incubated at 37 C. for three days in bell jars in which 10 per cent of the air was replaced by carbon dioxide. After the finding of Kiggins and Plastridge[3] that the optimum gaseous environment for growth of *V. fetus* consisted of 5 per cent oxygen, 10 per cent carbon dioxide, and 85 per cent nitrogen, the jars were evacuated to 25 per cent of atmospheric pressure; 10 per cent carbon dioxide and 65 per cent nitrogen were then added. The optimum gaseous atmosphere is easily obtained with the aid of a manifold tube with suitable connections to the jar, commercial tanks of gas, vacuum pump, and manometer.

Following incubation, the plates were examined with a x 12 binocular dissecting microscope using an oblique light source. Small, translucent, light gray colonies (0.3 to 1.0 mm. in diameter) were transferred to thiol medium (2 to 5 days after preparation). Up to 15 colonies were subcultured from the plates, depending upon the number available.

The subcultures were incubated five days in a jar in which 10 per cent of the air was replaced with carbon dioxide. All cultures that showed growth characteristic for *V. fetus* (growth band in the upper 5.0 mm. of medium) were examined under a phase-contrast microscope. If motile and comma- or spiral-shaped rods were present, a transplant for further study was made and the remainder of the culture was tested for catalase content. This was done by mixing 5.0 ml. of the culture with 5.0 ml. of a 3 per cent solution of hydrogen peroxide in a Smith fermentation tube. The appearance of more than 5.0 mm. of oxygen in the tube, after 30 minutes at room temperature, was interpreted as positive and the culture was classed as *V. fetus*.

Agglutination antigens were prepared from 110 of the catalase-positive and 11 of the catalase-negative Vibrio cultures. The antigens were tested against antiserums for two serological types of

From the Department of Animal Diseases, Storrs Agricultural Experiment Station, University of Connecticut, Storrs.

Supported in part by funds provided for regional research by the Research and Marketing Act of 1946 under project NE-1, by the New England Artificial Breeding Council, and by Lederle Laboratories Division, American Cyanamid Co., Pearl River, N.Y.

The writers are indebted to Messrs W. R. Amidon, P. Browne, F. A. Buschner, and P. Heller for supplying the bovine semen samples.

TABLE 1—Incidence of Carrier Bulls

AI center	No. of bulls			Percentage positive
	Total	Negative	Positive	
1	30	22	8	27
2	38	25	13	34
3	37	22	15	40
4	44	19	25	57
Total	149	88	61	41

V. fetus. The antigens were prepared from cells grown on thiol agar slants. The slants were inoculated by streaking the surface with several loopfuls of the thiol culture and were incubated for three days in the optimum gaseous environment for *V. fetus.* The cells were then suspended in Sorensen's buffer solution (pH 7.0) that contained 0.3 per cent formalin. The suspension was adjusted to a density of 1.5 on the McFarland nephelometer scale.

Blood samples from 52 bulls were examined for *V. fetus* agglutinins. The blood samples were collected within a period of two months before or after the time the cultural tests were made.

RESULTS

Influence of Gaseous Environment on the Isolation of Vibrio Fetus.—Duplicate sets of blood agar plates were inoculated with semen from 41 bulls that were found to be carriers on this or subsequent tests. One set was incubated in jars in which 10 per cent of the air was replaced with carbon dioxide. The second set was incubated in jars in which the optimum gaseous atmosphere for *V. fetus* was provided. Colonies that resembled *V. fetus* were isolated from both sets of plates and identified.

Vibrio fetus was isolated from 22 (54%) of the 41 samples when the optimum gaseous environment was used, as compared with only 8 (20%) when the atmosphere consisted of 10 per cent carbon dioxide and 90 per cent air.

Incidence of Carrier Bulls.—The percentage of carrier bulls in four AI centers is shown (table 1).

Number of Tests to First Positive Finding.—The number of tests on semen that were required to detect *V. fetus* in 61 bulls is given (table 2).

TABLE 2—Tests Required to Detect Vibrio Fetus

AI center	No. of bulls	Percentage positive on test				
		1	2	3	4	5
1	8	25	12	50	12	....
2	13	38	38	23	....	....
3	15	47	20	33	....	....
4	25	40	32	20	4	4
Total	61	39	28	28	3	2

The percentage of carrier bulls found positive on the first test was 39 per cent; on two tests, 67 per cent; and on three tests, 95 per cent. The low percentage found on the first and second tests on the bulls in AI center 1 appears to be due to holding some lots of samples for periods longer than 48 hours before examination.

The results indicate that, for practical purposes, three tests are necessary before concluding that a bull is free from *V. fetus.*

Repeated Tests on Carrier Bulls.—*Vibrio fetus* was isolated from 143 (54%) of 263 semen samples from 57 bulls. From two to 13 tests were made on individual bulls. The first six tests, on bulls that were tested more than six times, were used in compiling the results (table 3).

While *V. fetus* was isolated from 54 per cent of the samples, the ratio of positive to total samples varied for individual bulls. For example, 2 bulls were positive on one of six tests and 2 bulls were positive on six of six tests.

Isolation of Vibrio Fetus from Semen.—A total of 544 semen samples from 149 bulls in four AI centers were examined; 145 (27%) yielded *V. fetus,* 17 (3%) yielded catalase-negative vibrios, 321 (59%) were negative for vibrios, and 61 (11%) contained bacteria which overgrew the plates and prevented a satisfactory test for *V. fetus.*

The amount of semen streaked on the blood agar plates was selected after preliminary trials with different amounts. Plate 1 was streaked with 0.01 ml. of

TABLE 3—Repeated Tests* on Carrier Bulls

No. of bulls	No. of tests on each bull	Positive/total tests
9	2	1/2
3	2	2/2
9	3	1/3
2	3	2/3
1	3	3/3
3	4	1/4
3	4	2/4
1	4	3/4
1	4	4/4
2	5	1/5
1	5	2/5
5	5	3/5
2	6	1/6
2	6	2/6
4	6	3/6
2	6	4/6
5	6	5/6
2	6	6/6

* Includes the first six tests, only, for bulls tested more than six times.

TABLE 4—Isolation of Vibrio Fetus

Plate		*V. fetus* isolated	
1	2	No.	%
N	P	39	27
P	N	51	35
P	P	55	38

N = negative; P = positive.

semen. When larger amounts were used, the plates were usually overgrown with colonies of saprophytic bacteria. At times, plate 1 was overgrown when the inoculum was 0.01 ml. For this reason, a 1:30 dilution of semen was prepared and 0.02 ml. streaked on the surface of plate 2. The rate of isolation of *V. fetus* from the two plates is shown (table 4).

Use of plate 2 resulted in isolation of *V. fetus* from 27 per cent of the infected samples that failed to yield *V. fetus* colonies on plate 1 owing to heavy growth of saprophytic bacteria.

The number of colonies per sample that resembled *V. fetus* averaged eight and varied from one to 15. The proportion of such colonies that were identified as *V. fetus* averaged one of three, and ranged from one of 13 to eight of eight. Diphtheroids and a motile unidentified gram-negative rod were the principle bacteria responsible for colonies that resembled *V. fetus*. These organisms were usually present in fairly large numbers in semen from negative as well as *V. fetus*-positive bulls.

The estimated number of *V. fetus* organisms present in semen from which the organism was isolated averaged 800, and ranged from 100 to 9,000 per milliliter. Negative tests obtained on bulls which were positive on one or more tests indicate that the number of *V. fetus* organisms present in the semen of carrier bulls is frequently less than 100 per milliliter.

Isolation of Vibrio Fetus from Preputial Washings.—A total of 18 samples were obtained from 12 bulls that yielded positive semen within a period of two months, before or after, the collection of preputial samples. Of the 18 samples, seven (39%) yielded *V. fetus*. Of the seven positive samples, *V. fetus* was obtained from four before and from six after centrifugation. None of nine preputial samples from seven bulls with negative semen yielded *V. fetus*.

The proportion of Vibrio-like colonies obtained from preputial washings that were identified as *V. fetus* was one of 17 for the uncentrifuged samples and one of six for the centrifuged portions.

Culturing of preputial washings gave a lower percentage of positive findings than culturing of semen.

Low speed centrifugation of the preputial samples increased the chances of isolating *V. fetus* by reducing the number of saprophytic bacteria. However, centrifugation of semen was not advantageous, presumably because the viscosity and specific gravity of semen is higher than that of the tryptose broth used in the collection of preputial washings.

Identification of Vibrio Fetus.—The majority of diphtheroids that produced Vibrio-like colonies failed to grow when transferred to thiol medium. However, a few diphtheroids and an unidentified gram-negative rod produced growth in thiol medium that resembled that of *V. fetus*. Such cultures in thiol medium were catalase positive. Consequently, microscopic examination of *V. fetus*-like growth in thiol medium is necessary.

Differentiation of *V. fetus* from other vibrios found in the samples examined was readily accomplished by use of the catalase test, as reported by Bryner and Frank.[1] The 16 catalase-negative vibrios produced less than 5 mm. of oxygen. In addition, the majority produced two growth zones in thiol medium—one a layer of growth 1 to 2 mm. beneath the surface (like *V. fetus*) and the other a weak diffuse zone of uniform turbidity extending about 2 cm. downward from the layer of dense growth. Of the 145 catalase-positive cultures, 3 per cent produced 10 mm. of oxygen; 6 per cent, 20 mm.; 78 per cent, 35 to 50 mm.; and 13 per cent, over 50 mm. Tests for catalase production should be made on cultures that show good growth. Occasionally, recently isolated cultures require several transfers before good growth is obtained.

Agglutination tests were made on 110 catalase-positive cultures of *V. fetus* and 11 catalase-negative cultures of vibrios. Of the 110 cultures regarded as *V. fetus*, 51 (46%) were agglutinated by antiserum against the predominant type of *V. fetus* (type 1), four (4%) by type 2 antiserum, 1 (1%) was spontaneously agglutinable, and 54 (49%) were inagglutinable. Whether the inagglutinable cultures repre-

sent other antigenic types or were inagglutinable strains belonging to types 1 or 2 remains to be determined. None of 11 catalase-negative cultures were agglutinated by the two types of *V. fetus* antiserums.

Blood Serum-Agglutination Tests on Bulls.—Two, only, of 21 culturally positive bulls reacted positively (titers of 1:50 and 1:100), respectively. One of 31 culturally negative bulls gave a titer of 1:50. With the antigen used, a titer of 1:50 or higher was interpreted as positive.

DISCUSSION

The high incidence of *V. fetus*-carrier bulls in AI centers, as reported here and by Hughes,[2] and failure of the blood serum-agglutination test to detect a significant proportion of such bulls shows the need for testing semen and preputial washings by cultural methods. While the use of blood agar plates as described herein has limitations, it has advantages over culturing in a fluid medium. Its limitations are that it fails to detect fewer than 100 *V. fetus* organisms per milliliter of semen, and *V. fetus* colonies are visibly indistinguishable from colonies of certain saprophytic bacteria, particularly those of diphtheroids. Use of a larger inoculum than that used (0.01 ml.) for the purpose of detecting small numbers of *V. fetus* was found to be impractical because of heavy growth of saprophytic bacteria.

In attempting to evaluate therapeutic measures, a series of tests should be made before treatment, as well as after, in order to establish the frequency of isolation of *V. fetus* from the bulls scheduled for treatment. In the present investigation, the rate of isolation of *V. fetus* varied from one of six tests to six of six tests. In addition, before treatment of carrier bulls in AI centers is attempted, a sanitary program to prevent the transfer of *V. fetus* to negative and treated bulls is necessary. The high incidence of bulls that carry *V. fetus* leaves little doubt that present methods of collecting semen result in the transmission of *V. fetus*.

The absence of agglutinins in the blood serum of most bulls that yielded semen containing *V. fetus* supports the opinion that *V. fetus* has little, if any, pathogenicity for the bull, and that *V. fetus* exists primarily as a saprophyte in the genitourinary tract of carrier bulls.

SUMMARY

A procedure for detecting bulls which carry *Vibrio fetus* by culturing semen and preputial washings on blood agar, incubating the plates in the optimum gaseous environment for growth of *V. fetus*, and isolating and identifying the organism is described.

The results obtained on 149 bulls in four artificial insemination (AI) centers showed that:

1) The incidence of carrier bulls was 41 per cent.

2) The percentage of carrier bulls found positive by one, two, and three tests was 39, 67, and 95 per cent, respectively.

3) In repeated tests on 57 carrier bulls, 54 per cent of 263 semen samples yielded *V. fetus*.

4) Of a total of 544 semen samples from 149 bulls, 27 per cent yielded *V. fetus*, 3 per cent catalase-negative vibrios, 59 per cent were negative for *V. fetus*, and 11 per cent contained saprophytic bacteria, either "spreading" colony producers or excessive numbers, that prevented a satisfactory test.

5) The number of colonies per semen sample that resembled those of *V. fetus* varied from one to 15. The proportion of such colonies that were identified as *V. fetus* averaged one of three, and ranged from one of 13 to eight of eight.

6) Seven of 18 preputial samples from 12 carrier bulls were positive. Centrifugation of preputial washings at 1,000 g for 20 minutes and culturing of the supernatant fluid increased the chances of isolating *V. fetus*.

7) Serological tests made on antigens of 110 cultures of *V. fetus* showed that 51 belonged to type 1 (most common type found in cattle), four to type 2, one was spontaneously agglutinable, and 54 were inagglutinable by the antiserums used. None of the antigens of 11 catalase-negative vibrios were agglutinated by the *V. fetus* antiserums.

References

[1] Bryner, J. H., and Frank, A. H.: A Preliminary Report on the Identification of *Vibrio Fetus*. Am. J. Vet. Res., *16*, (1955): 76–78.

[2] Hughes, D. E.: Notes on Vibriosis, with Special Reference to the Isolation of *Vibrio Fetus* from Semen and Preputial Fluids. Cornell Vet., *46*, (1956): 249–256.

[3] Kiggins, E. M., and Plastridge, W. N.: Effect of Gaseous Enviroment on Growth and Catalase Content of *Vibrio Fetus* Cultures of Bovine Origin. J. Bact., *72*, (1956): 397–400.

Observations on Experimental Bovine *Vibrio Fetus* Infection

HANS BLOBEL, Dr. med. vet.; J. SIMON, D.V.M., Ph.D.; S. H. McNUTT, D.V.M.

Madison, Wisconsin

IN THIS STUDY, *Vibrio fetus* infection was experimentally induced in cattle to further characterize the disease and to obtain a more comprehensive evaluation of diagnostic procedures. The literature on bovine genital vibriosis was recently reviewed by Boyd [1] and by Steger.[2]

MATERIALS AND METHODS

Experimental Animals.—Twenty-two heifers, 14 to 18 months of age, of an isolated, brucellosis-free herd, mostly Holstein-Friesians, were employed in this study. Animals were selected at random. Seven of these heifers, group A, were exposed vaginally with *V. fetus* cultures in May, 1955. Another 7 heifers, group B, were inoculated with suspensions of 1.0 cc. of diluted semen and 10.0 cc. of *V. fetus* cultures intrauterinely via catheter on the day of heat. One pregnant heifer was fed 40.0 cc. of a suspension of mixed strains of *V. fetus* on two consecutive days. The remaining 7 animals, the control group, were artificially inseminated with 1.0 cc. of diluted semen from bulls presumed to be free of vibriosis.

Vibrio Fetus Cultures.—Five catalase-positive strains of *V. fetus* were employed. They were isolated from bull semen and aborted fetuses in Wisconsin from 1951 to 1953. The strains were maintained in Albimi * semisolid medium. Each strain was subcultured every two to three weeks.

The antigen for the agglutination test was prepared essentially as described by Ristic *et al.*[3] with the modification of the microaerophilic conditions. Helium or nitrogen were used as a diluent rather than carbon dioxide.[4]

The final test antigen was adjusted to a light transmissibility of 50 per cent in a Coleman Nepho-colorimeter with a 655-mμ filter.

Testing Procedures.—Vaginal mucus samples were taken weekly from all heifers. They were collected and prepared for testing, as described in detail by Hughes.[5] Blood was drawn from the animals of group B, from the heifer exposed orally to *V. fetus* organisms, and from the control animals at biweekly intervals. A twofold serial dilution of the vaginal mucus and the blood serum was tested against the homologous and four heterologous *V. fetus* antigens. The diluent used for the mucus was 0.3 per cent formalized physiological saline and all dilutions were made with an automatic Cornwall pipette. As a control, 0.5 cc. of 0.3 per cent formalized physiological saline was mixed with 0.5 cc. of the respective antigens. The tubes were then incubated at 37 C. and the tests read 48 hours later. The readings were recorded as the reciprocals of the highest dilution showing complete agglutination. This was evidenced by complete clearing and visible clumps of agglutinated cells when the tube was gently tilted.

Isolation of Vibrio Fetus.—The medium most frequently used for the isolation of *V. fetus* from infected animals was Difco blood agar base † medium in Petri plates. Additional cultures were made on Albimi semisolid agar * or Difco semisolid thiol medium† as suggested by Huddleson.[6] The sterilized semisolid mediums were kept in pyrex glass tubes. These tubes were stoppered with tightly rolled cotton plugs. Each sample to be cultured was inoculated into at least six, and frequently into eight or ten, of the respective plates or tubes of mediums.

RESULTS

Laboratory Studies.—Prior to the experimental exposure, the vaginal mucus of none of 22 heifers contained agglutinins to the five *V. fetus* strains employed in this study. The 7 animals of group A were exposed vaginally to *V. fetus* in May, 1955. Three animals of this group, namely, 10N, 13N, and 14N, were pregnant at the time of exposure.

Five heifers (71.4%) of group A developed agglutinins to *V. fetus* in the vaginal mucus four to ten weeks after the experimental infection. These were the heifers 6N, 10N, 13N, 7S, and 14N (graph 1). The agglutinins could be demonstrated with the homologous and four heterologous antigens. The vaginal mucus agglutinin titers reached their peaks approximately ten to 12 weeks after exposure and receded gradually thereafter. Animals 10N and 13N calved normally and *V. fetus* could not be reisolated.

Most interesting results were obtained

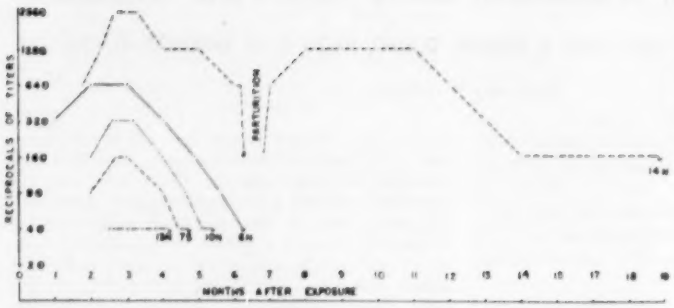
From the Department of Veterinary Science, University of Wisconsin, Madison.

Paper 212 published with the approval of the director of the Wisconsin Agricultural Experiment Station. Supported in part by a grant from the Research Committee of the Graduate School from funds provided by the Wisconsin Alumni Research Foundation.

This is a condensation of a thesis which was submitted by H. Blobel in partial fulfillment of the requirements for the M.S. degree, University of Wisconsin, 1956.

* Albimi Laboratories.

† Difco Laboratories, Detroit, Michigan.



Graph 1—Agglutination of homologous Vibrio fetus antigens by vaginal mucus from heifers 6N, 10N, 13N, 7S, and 14N.

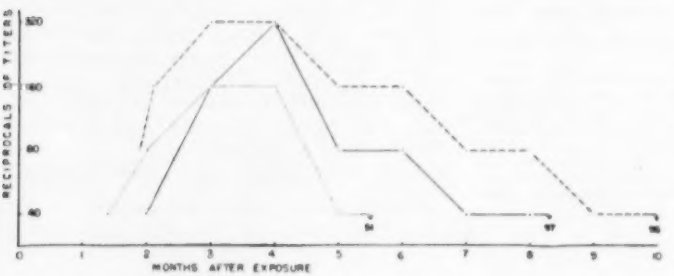
from heifer 14N. Her vaginal mucus contained agglutinins for *V. fetus* in a titer of 1:2,560 for three weeks starting approximately ten weeks after the experimental infection. The vaginal mucus of this animal agglutinated homologous and heterologous *V. fetus* cells in dilutions to 1:1,280 at all subsequent testing periods. She calved normally on Nov. 21, 1955, and had a live calf. On Dec. 5, 1955, two weeks after calving, cow 14N continued to show agglutinins in the vaginal mucus which reached a maximum titer of 1:1,280 approximately five weeks after parturition. Then, the titer gradually receded. In November, 1956, 18 months after the experimental infection, her vaginal mucus still agglutinated homologous and heterologous *V. fetus* cells in dilutions up to 1:160. The blood serum agglutinin titers of this animal with the homologous antigen remained constant at 1:20, before and after calving. Several attempts to demonstrate *V. fetus* agglutinins in the whey were unsuccessful. *Vibrio fetus* organisms could be recovered from the dirty yellowish, odorless uterine discharge until the ninth day after calving and not again until approximately five months after parturition, April 30, 1956, when cow 14N was in heat. In previous and subsequent estrous periods which occurred at prolonged and irregular cycles, *V. fetus* could not be reisolated.

The conjunctival administration (0.5 cc.) and intradermal injection (0.1 cc.) of the homologous concentrated *V. fetus* antigens did not elicit any conclusive sensitivity reactions in the experimentally infected animals.

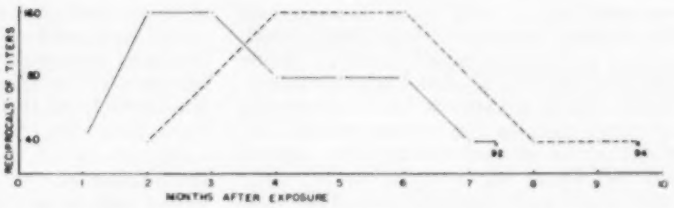
In the case of the pregnant heifer which was fed *V. fetus* cultures, an agglutinin response could not be detected in the vaginal mucus or blood serum for six months following exposure. The animal calved normally and *V. fetus* organisms could not be recovered.

The 7 heifers of group B were inoculated intrauterinely with diluted semen and *V. fetus* cultures at estrus. The experiment started in September, 1955. Five animals (71.4%) of group B contained agglutinins in the vaginal mucus four to nine weeks after the experimental infection with *V. fetus*. These were the heifers 91, 95, 97 (graph 2), and 92 and 94 (graph 3). The titers ranged from 1:40 to 1.640 and persisted for approximately six to ten months. The titers obtained with the respective homologous and the four heterologous *V. fetus* antigens were essentially similar. None of these animals aborted.

There was a considerable variation in



Graph 2—Agglutination of homologous Vibrio fetus antigens by vaginal mucus from heifers 91, 95, and 97.



Graph 3—Agglutination of homologous Vibrio fetus antigens by vaginal mucus from heifers 92 and 94.

the blood serum agglutinin titers of the individual heifers, depending on the strain of *V. fetus* employed as antigen. With one antigen, the maximum blood serum titer was 1:640, with another 1:20 in the same animal. Only 2 animals of group B (28.6%) showed a rise in these blood serum titers against the homologous and only slightly against the heterologous *V. fetus* antigens. In 1 heifer, the blood serum titers rose from 1:20 to 1:80 and in the other from 1:80 to 1:1,280. The rise in titer began in 12 days and reached its maximum six weeks after the experimental infection. The vaginal mucus of both heifers agglutinated *V. fetus* cells. Their vaginal mucus titers persisted over a longer period of time than the increased agglutinin level in the blood serum.

In the vaginal mucus of the control animals, agglutinins to the *V. fetus* strains used for this study could not be detected for approximately six months. However, agglutinins to *V. fetus* were demonstrable in the blood serums of all control animals. The titers varied from 1:20 to 1:640, depending on the strains of *V. fetus* employed as antigens. They were essentially the same as the corresponding values obtained from the animals of group B, with the exception of the 2 heifers whose blood serum titers rose during the course of the experimental infection.

Service Records.—Two heifers of group B conceived after the first service and carried normal calves to term. The vaginal mucus and the blood serum agglutination tests of both animals proved negative throughout.

In group B, heifer 92 conceived after two, heifers 91 and 97 after three, heifer 95 after four, and heifer 94 after six inseminations. All these animals exhibited irregular and prolonged estrous cycles, ranging from 25 to 43 days, and developed agglutinins in the vaginal mucus. Thus, the 5 animals of group B with demonstrable vaginal mucus agglutinins required an average of three and six-tenths inseminations per pregnancy. In the 7 control animals with no detectable vaginal mucus agglutinins, an average of approximately one and four-tenths inseminations per pregnancy were needed.

DISCUSSION

It is recognized that the preceding findings are limited and could be influenced by the *V. fetus* strains employed. Their virulence and other properties could have been altered, since these strains were maintained for over two years on laboratory mediums prior to use in this study. This should be borne in mind when evaluating the results of these experiments. Changes in virulence, particularly in invasiveness, may also explain, if we neglect natural resistance, that only 71.4 per cent of the heifers exposed to a relatively large dose of live cells exhibited evidence of infection and did not abort. However, these data gathered in this study of experimental bovine *V. fetus* infection may be helpful, particularly in evaluating current diagnostic procedures.

The most reliable method for diagnosing the disease has been the isolation and identification of the organisms from the reproductive organs or from aborted fetuses and their membranes. However, isolations of *V. fetus* often fail, as the organism is relatively fastidious and is readily overgrown by other microbes, especially by species of the genera Pseudomonas and Proteus. In addition to this, suitable specimens for the bacteriological procedure are not always obtainable. Therefore, an attempt has been made to correlate bacteriological results and characteristic clinical findings with demonstrable antibodies against *V. fetus*.

The vaginal mucus agglutination test has been widely used as an aid in the diagnosis of bovine vibriosis for the past few years. Terpstra and Eisma [7] and Sjollema [8] indicated the practical diagnostic value of the vaginal mucus agglutination test when

employed on a herd basis. Lawson and MacKinnon [9] concluded from field investigations and experimentally induced bovine vibriosis that the *V. fetus* mucus agglutination test is a valuable aid to diagnosis, provided samples for testing are not collected at or around estrus. The copious flow of mucus at estrus tends to dilute agglutinins and this produces erratic results. McEntee *et al.*,[10] Hughes,[5] Boyd,[1] Easterbrooks *et al.*,[11] Plastridge *et al.*,[12] and others recognized also the diagnostic significance of detectable *V. fetus* agglutinins in the vaginal mucus when properly evaluated. Most recently Vandeplassche *et al.*[13] and Steger [2] stated that the vaginal mucus agglutination test proved to be a valuable diagnostic aid.

In this study of experimental *V. fetus* infection in cattle, significant vaginal mucus agglutinin titers could be distinctly correlated with the "repeat-breeding syndrome" and, in the case of animal 14N, with the repeated recovery of *V. fetus* organisms. This indicates the practical diagnostic significance of the vaginal mucus agglutination test.

Smith *et al.*[14] suggested the blood serum agglutination test as a diagnostic aid in bovine vibriosis. They found that the blood serum of 3 cows which aborted from vibriosis agglutinated *V. fetus* organisms. Barger [15] reported similar findings for 2 cases of vibrionic abortions. Following this, the blood serum agglutination test had been studied by various investigators for many years, but its efficacy for the diagnosis of bovine vibriosis is still controversial.

In this experiment, blood serum agglutinin titers of the individual heifers varied considerably with the strain of *V. fetus* employed as antigen. The titers obtained were essentially the same in the control animals and in the animals exposed to *V. fetus* cultures with the exception of 2 animals of group B. Both animals exhibited a temporary rise in these blood serum titers against the homologous and only partially against the heterologous *V. fetus* antigens. This is essentially in concordance with the observations made by Simon.[16] He reported that "the maximum blood serum titer in a cow was 1:40 when one antigen was employed, 1:640 with another." Simon [16] found that "the blood serum titers obtained with any one antigen varied from cow to cow." In the case of animal 14N, the blood serum agglutinin titers, even with the homologous antigen, remained constant at 1:20 before and after calving. *Vibrio fetus* could be repeatedly reisolated from this animal. She also reacted positively to the vaginal mucus test.

In view of all these findings, the practical value of the blood serum agglutination test in diagnosing bovine vibriosis appears to be doubtful. It will be especially difficult to determine the "infectious" titers of blood serums in field investigations.

Generally, the results obtained in this study indicate that vibriosis appears to be essentially a localized genital infection in cattle. This is in concordance with the findings of Terpstra and Eisma,[7] Stegenga and Terpstra, [17] Lawson and MacKinnon,[9] and others. All these authors found evidence primarily of vibriosis in the reproductive organs of cattle. This would explain why out of 6 infected animals with positive vaginal mucus titers, only 2 manifested a temporary rise in blood serum titers with the respective homologous antigens during the course of the infection. This, also, would account for the absence of agglutinins against *V. fetus* in the milk of the known infected cow 14N. This animal had a vaginal mucus agglutinin titer of 1:1,280 and *V. fetus* organisms could be isolated from the uterine discharge at the time the milk samples were taken. The assumption that there is usually only a local immunological response would explain, too, the failure to detect a sensitivity reaction upon conjunctival administration and of intradermal injection of the homologous antigen suspension.

SUMMARY

The following observations were made in this study of experimental *Vibrio fetus* infection in cattle:

1) Vibriosis could be produced by vaginal as well as uterine exposure in 10 (71.4%) of 14 heifers. The oral inoculation of *V. fetus* cultures in 1 pregnant heifer did not result in infection.

2) Evidence of infection was based upon the "repeat-breeding syndrome," the recovery of *V. fetus* organisms from the uterine discharge, and the demonstration of specific agglutinins in the cervicovaginal secretions.

3) There was a distinct correlation be-

tween detectable vaginal mucus agglutinins and lowered reproductive efficiency. *Vibrio fetus* could be repeatedly recovered from animal 14N whose vaginal mucus agglutinin titers reached 1:2,560.

4) The vaginal mucus agglutination test proved to be a valuable aid in the diagnosis of bovine vibriosis, provided samples for testing were not collected at or near estrus. The interval required for the development of detectable levels of agglutinins in the cervicovaginal secretions following exposure was four to ten weeks. The vaginal mucus titers persisted for approximately four to 12 months.

5) It appeared that the practical diagnostic value of the serum agglutination test is doubtful. Agglutinins to *V. fetus* could not be demonstrated in the milk of a known infected cow. The conjunctival administration and intradermal injection of homologous antigens did not elicit any conclusive reactions.

References

[1] Boyd, H.: Bovine Genital Vibriosis. Academic disputation, Royal Vet. Coll., Stockholm, Sweden, 1955.

[2] Steger, G.: Beobachtungen zur Vibriosis Genitalis beim Rind. Fortpflanzung, Zuchthygiene und Haustierbesamung. Heft, *4*, (1956): 49.

[3] Ristic, M., Sanders, D. A., and Tyler, M. E.: *Vibrio Fetus* Infection in Cattle: A Comparative Study of the Agglutinin Response in Cervicovaginal Mucus and Blood Serum to Homologous Antigens Following Abortion. Am. J. Vet. Res., *59*, (1955): 246.

[4] Reich, C. V., Morse, E. V., and Wilson, J. B.: Gaseous Requirements for Growth of *Vibrio Fetus*. Am. J. Vet. Res., *17*, (1956): 140.

[5] Hughes, D. E.: A Study of the Diagnosis of Bovine Vibriosis with Special Reference to the Detection of Agglutinins in the Vaginal Secretions. Cornell Vet., *3*, (1953): 431.

[6] Huddleson, J. F.: A Satisfactory Medium for the Isolation, Cultivation and Maintenance of Viability of *Vibrio Foetus* (Bovine). J. Bact., *56*, (1949): 508.

[7] Terpstra, J., and Eisma, W. A.: *Vibrio Foetus* in Cattle and Enzootic Infertility. Tijdschr. Diergeneesk., *75*, (1950): 433.

[8] Sjollema, P.: *Vibrio Foetus* Infection in Cattle and Its Influence on Fertility. Internat. Congr. Physiol. and Path. Anim. Reproduction, Copenhagen, *3*, (1953): 35.

[9] Lawson, J. R., and MacKinnon, D. J.: *Vibrio Fetus* Infection in Cattle. Vet. Rec., *64*, (1952): 763.

[10] McEntee, K., Hughes, D. E., and Gilman, H. L.: Experimentally Produced Vibriosis in Dairy Heifers. Cornell Vet., *44*, (1954): 376.

[11] Easterbrooks, H. L., Plastridge, W. N., Williams, L. F., and Kiggins, E. M.: Vibriosis: Diagnostic and Therapeutic Considerations. Vet. Med., *2*, (1955): 51.

[12] Plastridge, W. N., Easterbrooks, H. L., Williams, L. F., Kiggins, E. M., and Walker, E.: Reproductive Efficiency and Serological Findings in Heifers Experimentally Infected with *Vibrio Fetus*. Am. J. Vet. Res., *16*, (1955): 493.

[13] Vandeplassche, M., Paredis, F., Van Niewenhuyse, E., Brone, E., and Florent, A.: Beitraege zur *Vibrio Fetus*-Infektion bei Rindern mit besonderer Berücksichtigung der Unfruchtbarkeit. Zentralbl. Vet. Med., *3*, (1956): 111.

[14] Smith, T., Little, R. B., and Taylor, M. S.: Further Studies on the Etiological Role of *Vibrio Fetus*. J. Exptl. Med., *32*, (1920): 683.

[15] Barger, E. H.: Report of a Case of Abortion Induced by *Vibrio Fetus*. J.A.V.M.A., *72*, (1928): 468.

[16] Simon, J.: Histopathological Alterations of the Bovine Uterus. Ph.D. thesis, University of Wisconsin, 1951.

[17] Stegenga, Th., and Terpstra, L. J.: Over *Vibrio fetus* Infecties bij het rund en "Enzootische steriliteit." Tijdschr. Diergeneesk., *74*, (1949): 293.

Parenteral Benzathine Penicillin V in Cattle

C. J. HOLLISTER, D.V.M.; R. A. HUEBNER, V.M.D.;
WM. B. BOUCHER, V.M.D.; THOMAS DeMOTT, V.M.D.

Philadelphia, Pennsylvania

Of the several different natural penicillins produced as the result of research studies, penicillin G (benzyl penicillin) is the most commonly used type. In the free acid form, it is highly unstable thus it is therapeutically useful primarily in the form of its stable soluble salts (such as sodium, potassium, procaine, etc.).

Penicillin V (phenoxymethyl penicillin) is relatively nontoxic and is essentially similar in antimicrobial activity to penicillin G. In the crystalline free acid form, it is insoluble and stable in acid medium, but it is highly soluble and readily absorbable from the alkaline upper intestinal tract. Jaksch *et al.*[1] concluded that orally administered penicillin V is effective in horses, hogs, dogs, and poultry. Huebner *et al.*[2] reviewed the literature, human and veterinary; they reported on the blood levels following oral doses of penicillin V in dogs and of penicillin V and benzathine penicillin V in poultry. Essentially, either penicillin V or its benzathine salt by the oral route produce higher and less variable penicillin blood serum levels than do salts of penicillin G.

Comparison between benzathine penicillin G and more soluble penicillin G salts, in various vehicles, by the oral and intramuscular routes, has been documented in the literature[3-9]; fundamentally, the benzathine salt produces more prolonged but lower penicillin blood levels.

This study was undertaken to determine the comparative response to parenteral administration of benzathine penicillin V, benzathine penicillin G, and procaine penicillin G.

Drs. Hollister, Boucher, and DeMott are on the staff of the School of Veterinary Medicine, University of Pennsylvania, Philadelphia, and Dr. Huebner is director, Department of Veterinary Service, Wyeth Laboratories, Philadelphia.

This study was supported, in part, by a research grant from Wyeth Laboratories Division of American Home Products Corp., Philadelphia.

The authors express their gratitude to Dr. William F. Elias, Mr. H. J. Merrion, Mr. M. K. Chelland, Miss Virginia Quinn, and Miss Frances Cisick of the Antibiotic Division, Wyeth Laboratories, West Chester, Pa., for their technical assistance in completing the blood and milk assays.

Materials and Methods

Blood serum samples from 20 normal, adult, milking cows of known background were examined immediately prior to injection and were found to be negative for any bacteriostatic activity. Ten animals were given a single intramuscular injection of 3,000,000 units benzathine penicillin V in aqueous suspension (10-cc. volume), 5 were given a similar injection of 3,000,000 units benzathine penicillin G, and 5 were given a similar injection of procaine penicillin G. Details on each animal's physical status, postinjection intervals at which blood and milk samples were drawn, and the assays of the penicillin content of these body fluids are summarized in table 1. Penicillin concentrations were determined by the microbiological cup-plate method using *Sarcina lutea* as the test organism.

Results and Discussion

A single injection of the compounds under study resulted in penicillin blood serum levels, the height of which appears to be directly related to solubility of the salt, with the ranking of (1) procaine penicillin G, (2) benzathine penicillin V, and (3) benzathine penicillin G. Duration of the levels was in inverse ratio to solubility, thus ranking (1) benzathine penicillin G, (2) benzathine penicillin V, and (3) procaine penicillin G.

More interesting, however, are the milk samples. In these secretions, benzathine penicillin V produced penicillin concentrations that were higher, longer, and more nearly uniform than those resulting from procaine penicillin G. Following benzathine penicillin V, penicillactia was approximately one third the penicillemia level; while after procaine penicillin G, it ranged from about one fifteenth to one fourth. These results suggest that penicillin reaches the milk from the blood stream not merely through a threshold mechanism but by a more specific involvement of the mammary tissues; too, there appears to be a difference in the mammary distribution rates between penicillin V and penicillin G.

Conclusions

Single, intramuscular doses of 3,000,000

TABLE 1—Penicillin Content of Body Fluids of Cows at Varying Times After Injection

Cow #	Wt. (lbs.)	Age (yrs.)	Lactating	Pregnant	Milk (lbs./day)	Units of Penicillin per cc. of Blood Serum									Units of Penicillin per cc. of Milk						
						Pre-injection	1 hour	6 hours	12 hours	24 hours	48 hours	72 hours	96 hours	120 hours	144 hours	1 hour	6 hours	12 hours	24 hours	48 hours	72 hours	96 hours
1a	800	5	6 Mo.	3 Mo.	28.9	0	0.075	-	0.128	0.098	0.049	0.020	0.032	0	0.070	0	0.032	0.053	0.052	0.028	0	0
2a	900	5	6 Mo.	3 Mo.	24.6	0	0.379	-	0.232	0.207	0.073	0.023	7.580	0	0	0	0.055	0.074	0.086	0.033	0	0
3a	850	4 1/2	3 Mo.	3 Wk.	29.3	0	0.245	0.144	0.100	0.096	0.049	0.021	0.047	0	0	0	0.041	0.052	0.037	0.028	0	0
4a	825	6 1/2	9 Mo.	5 Mo.	6.8	0	0.218	0.116	0.119	0.162	0.070	0.036	1.270	0	0	0	0.026	0	0.030	0	0	0
5a	870	8	13 Mo.	2 Mo.	14.7	0	0.245	0.105	0.105	0.207	0.162	0.041	0.024	0	0	0	0.019	0.024	0.038	0.028	0	0
6a	780	3	1 Mo.	Open	34.1	0	0.276	0.170	0.170	0.128	0.062	0.030	0.370	0	0	0	0.062	0.048	0.042	0.023	0	0
7a	810	5	2 Mo.	Open	36.9	0	0.650	0.130	0.119	0.160	0.055	0.023	0.020	0	0	0	0.064	0.072	0.038	0.036	0	0
8a	790	4	5 Mo.	1 Mo.	24.3	0	0.207	0.122	0.105	0.105	0.053	0.026	0.070	0	0	0	0	0.025	0.039	0.028	0	0
9a	750	2 1/2	2 Mo.	Open	28.5	0	0.270	0.280	0.207	0.160	0.079	0.027	0.060	0	0	0	0.039	0.053	0.067	0.040	0	0
10a	790	3 1/2	7 Mo.	4 Mo.	21.8	0	0.340	0.113	0.125	0.094	0.055	0.020	0.045	0	0	0	0.043	0.056	0.053	0	0	0
(a-Given single intramuscular dose 3,000,000 units (10 c.c.) benzathine penicillin V in aqueous suspension)					(Average)		0.290	0.147	0.141	0.142	0.071	0.027		0	0.007	0	0.038	0.046	0.048	0.024	0	0
11b	775	3	7 Mo.	4 Mo.	20.0	0	0.192	0.042	0.044	0.066	0.114	0.065	0.061	0.024	0.019	0	0	0	0	0.010	0	0
12b	850	6 1/2	7 Mo.	2 Mo.	18.5	0	0.122	0.044	0.060	0.083	0.098	0.065	0.050	0.024	0.020	0	0	0	0	0	0	0
13b	875	8	9 Mo.	5 Mo.	21.5	0	0.093	0.035	0.052	0.053	0.086	0.044	0.026	0.015	0	0	0	0	0	0	0	0
14b	825	5	6 Mo.	2 Mo.	19.0	0	0.066	0.030	0.038	0.091	0.065	0.083	0.042	0.025	0.019	0	0	0	0	0.015	0	0
15b	790	3	5 Mo.	6 Wk.	17.2	0	0.091	0.018	0.032	0.042	0.037	0.037	0.021	0.018	0.016	0	0	0	0	0	0	0
(b-Given single intramuscular dose 3,000,000 units (10 c.c.) benzathine penicillin G in aqueous suspension.)					(Average)		0.113	0.034	0.045	0.067	0.080	0.059	0.040	0.021	0.015	0	0	0	0	0.005	0	0
16c	925	6 1/2	10 Mo.	7 Mo.	8.3	0	0.400	0.660	0.530	0.143	0	0				0	0	0.062	0.035	0	0	
17c	880	7	8 Mo.	6 Mo.	17.5	0	0.350	0.550	0.300	0.050	0	0				0	0.033	0.047	0.012	0	0	
18c	1000	9	13 Mo.	4 Mo.	4.0	0	0.360	0.400	0.340	0.091	0	0				0	0.049	0.049	0.032	0	0	
19c	800	8	13 Mo.	5 Mo.	13.2	0	0.340	0.400	0.318	0.055	0	0				0	0.029	0.037	0.017	0	0	
20c	825	8	2 Mo.	Open	43.5	0	0.320	0.470	0.270	0.087	0.024	0				0	0.033	0.047	0.017	0	0	
(c-Given single intramuscular dose 3,000,000 units (10 cc.) procaine penicillin G in aqueous suspension.)					(Average)		0.354	0.496	0.352	0.085	0.005	0				0	0.029	0.048	0.023	0	0	

units of penicillin in aqueous suspension given to normal, adult, milking cows (800 to 900 lb. body weight) were followed by these results:

1) After benzathine penicillin V, there was significant penicillemia for 96 hours and significant and fairly uniform penicillactia for 48 hours.

2) After benzathine penicillin G, penicillemia persisted for 144 hours; penicillactia was negligible.

3) After procaine penicillin G, there was significant penicillema for 24 hours and significant but variable penicillactia for 24 hours.

Comparison of the results with these salts suggests that the development of penicillactia may result from active participation of the mammary gland rather than from a mere threshold mechanism of blood circulatory spill.

References

[1] Jaksch, W., Lunzer, J., Rabl, R. F., Stromer, F., and Gross, L.: Penicillin-Blutserumspiegelbestimmungen nach peroralen Gaben von Phenoxymethyl-Penicillin bei Pferd, Schwein, Hund und Huhn. (Penicillin Concentration in the Blood After Oral Administration of Phenoxymethyl Penicillin to Horses, Pigs, Dogs, and Fowl.) Wien. tierärztl. Monatschr., *41*, (1954): 687–708.

[2] Huebner, R. A., Mark, J. A., Skelley, J. F., Elias, W. F., and Merrion, H. J.: Phenoxymethyl Penicillin (Penicillin V). Univ. Pennsylvania Bull., Vet. Exten. Quart. 144, (1956): 20–30.

[3] Huebner, R. A., and Green, C. W. J.: Canine Penicillin Blood Levels Obtained with Oral Benzethacil. Univ. Pennsylvania Bull., Vet. Exten. Quart. 128, (1952): 138–141.

[4] Huebner, R. A.: Bovine Penicillin Blood Levels Obtained with Parenteral Benzethacil. Cornell Vet., *42*, (1952): 457–460.

[5] Glenney, W. C., Batory, C. F., and Huebner, R. A.: Canine Penicillin Blood Levels Obtained with Parenteral Benzethacil. Am. J. Vet. Res., *13*, (1952): 555–556.

[6] Kral, F., Huebner, R. A., and Blumner, H. N.: Ovine and Porcine Penicillin Blood Levels Obtained with Parenteral Dibenzylethylenediamine Dipenicillin G. Am. J. Vet. Res., *15*, (1954): 67–70.

[7] Hamilton, C. M.: N, N'-Dibenzylethylenediamine Dipenicillin G in Turkey Erysipelas. Poult. Sci., *33*, (1954): 629–632.

[8] Huebner, R. A., and Blumner, H. N.: Bovine Penicillin Blood Levels Obtained with Simultaneous Parenteral Administration of Dibenzylethylenediamine Dipenicillin G and Procaine Penicillin G. Cornell Vet., *44*, (1954): 56–60.

[9] Hollister, C. J., Huebner, R. A., Boucher, W. B., and DeMott, T.: Bovine Antibiotic Blood Levels Obtained with Antibiotics in Oil Suspensions. Am. J. Vet. Res., *16*, (1955): 391–393.

Physiological Aspects of Fluids in Female Genitalia with Special Reference to Cattle—A Review

DURWARD OLDS, D.V.M., Ph.D., and N. L. VanDEMARK, Ph.D.

Urbana, Illinois

INFERTILITY of cattle has long been an important source of economic loss to cattle breeders and a source of consternation to conscientious veterinarians and animal physiologists. It has been estimated that about 20.0 per cent of cows require three or more services per conception [227] and that 6.0 or 7.0 per cent are nonbreeders.[309] Studies of repeat-breeding cows and heifers [65, 294, 295] have indicated that failure of fertilization may occur in about 25.0 per cent of the animals. However, of the animals in which fertilization occurred, it was estimated that about 60.0 to 65.0 per cent of the embryos died within 34 days of conception. Gross abnormalities of the genital organs were observed in only 10.6 [295] and 13.5 per cent [294] of these repeat-breeding animals. Based on a study of reproductive organs from routinely slaughtered cows and heifers, Perkins *et al.*[232] estimated that about 3.3 per cent of the specimens showed abnormalities of sufficient magnitude to cause sterility, while 8.6 per cent had abnormalities which would be likely to lower fertility. This would indicate that perhaps about half of the infertile animals could be expected to show gross abnormalities. Thus, it appears certain that a large number of cows are infertile without showing gross evidence of abnormality.

The accurate diagnosis of the causes of infertility is both challenging and discouraging. On one hand, there are many known factors or conditions which can be successively excluded or confirmed during examination of herds or individuals. These factors include the infectious diseases, certain endocrine disorders, anatomic defects, nutritional deficiencies, and management practices.[226] On the other hand, little is known regarding the possible role played by variations in the physiological environment provided by the internal genitalia for spermatozoa, ova, and embryos.

The present review summarizes available information on the composition, significance, and control of luminal fluids in female genitalia. It is hoped that this compilation of published research will emphasize the need for further work and help to indicate areas where additional information would be particularly valuable.

ENDOCRINE CONTROL OF CELLULAR ACTIVITY

Estrogens have been shown to cause vasodilatation and increased vascularity of female genitalia.[118, 119, 130, 195, 196, 325] In general, dilatation of arterioles causes capillary pressure to rise, which, along with increased area of capillary walls and thinner walls, leads to greater permeability.[103] Many studies have shown that estrogen administration causes an increase in the water and ash content of the uterus and vagina.[9-13, 59, 69, 197, 293, 309, 335, 336]

Excellent reviews on the action of steroid hormones in uterine metabolism have been published by Roberts and Szego.[250, 292] The injection of estrogens is followed in a few hours by increased anaerobic and aerobic glycolysis in uterine tissue.[58, 76, 81, 114-115, 150, 155, 156, 160, 195, 246, 261, 290] Estrogens have been shown to stimulate synthesis of uterine ribonucleic acid in ovariectomized rats.[206] There is also an increase in adenosine triphosphatase (ATPase) activity [223] and in high energy phosphates.[224] Progesterone, on the other hand, has been found to decrease ATPase activity.[313] Estradiol-treated rats showed an increased ability to incorporate labeled glycine into protein.[214] Blood sugar of cows has been reported to increase during estrus.[78]

It has been observed that estrogen administration increases the *beta* glucuronidase activity of the uterus and vagina, while progesterone decreases this activity.[26, 97, 98, 122, 151, 152, 164, 180, 206] Increased activity of *beta* glucuronidase is associated with growth of the uterus. This enzyme

From the Department of Dairy Science, University of Illinois, Urbana.

This article is part of a dissertation submitted, while the senior author was an AVMA fellow at the University of Illinois, in partial fulfillment of the requirements for the Ph.D. degree.

Dr. Olds' present address is Dairy Section, University of Kentucky, Lexington.

may also be associated with the synthesis of mucins and with the metabolism of estrogens.[288]

The administration of estrogens has been shown to cause an increase in the alkaline phosphatase in uterine epithelium, glands, and circular muscle.[13,100,102,142,213,297,327] The phosphatases are known to take part in metabolism by catalyzing the hydrolysis of certain phosphate esters.[288] The synthesis of phosphate esters is dependent on the action of phosphorylases, not phosphatases.

The administration of estrogens to ovariectomized rats has been observed to cause pronounced peroxidase activity in the uterus.[190]

HISTOLOGICAL AND HISTOCHEMICAL FEATURES

Vagina.—The epithelium of the anterior vagina consists of columnar to cubical mucus-secreting types of cells, while that of the posterior vagina is composed of stratified squamous cells.[4] There is a congestion of the blood vessels and edema of the stroma during proestrus and estrus.[70,249] In the cow, columnar cells of the anterior vagina are tallest during heat,[70] but the remainder of the epithelium tends to be more squamous from estrus to ten days postestrus, although true cornification does not occur. In laboratory animals, mucification of vaginal epithelium appears to be produced by the simultaneous action of estrogen and progesterone.[79,162,163,207] Larger doses of estrogen cause cornification.[207] Ford[101] found that progesterone, in adequate dosage, would inhibit the cornifiying action of estrogen and cause mucification.

Lipoid substances have been found in vaginal smears of guinea pigs.[218] It was suggested that the shedding of vaginal epithelium at estrus may be a process of fatty degeneration. Intracellular mucoid is restricted to superficial epithelial cells and its elaboration is maximal during the proestrous stage.[298] Intracellular glycogen was found to reach a maximum concentration during proestrus and lipid was most abundant during proestrus and estrus. However, Vokaer[312] reported that the glycogen in vaginal epithelium does not change under different estrogen dosages.

Alkaline phosphatase activity of vaginal epithelium reaches a maximum during proestrus, under the influence of estrogens.[141,147,247,310] There may be an association between this phosphatase activity and pentose nucleic acid in the synthesis of fibrous protein (*i.e.*, keratin). Alkaline phosphatase has also been reported to increase in cervical mucus under estrogenic influence.[177]

Asscher and Turner[8] observed that sulfhydryl groups accumulated in the basal layers of mouse vaginal epithelium during diestrus. In the process of keratinization, sulfhydryl groups were oxidized to disulfide groups.

Vaginal smears from cows have been found to contain few cornified cells at estrus.[121,208] There are ordinary epithelial cells, large polyhedral cells (basal cells of epithelium?), small round cells from the cervix and uterus, and no leukocytes. There is a preponderance of leukocytes in the smears three hours after mating.[264] Otherwise, the presence of leukocytes in the smears during heat is suggestive of pathological conditions. There are conflicting opinions regarding the reliability of vaginal smears in diagnosing estrus or stage of cycle in the cow.[94,244,262,275]

Cervix.—Herrick[134] has reported that mucus-producing cells of the cervix in cows show maximum fullness at estrus. At this time, the luminal ends of the cells rupture and discharge their cytoplasm (mucus) into the lumen. Cesa[61] reported that mucous glands of the cervix were stimulated by estrogens and that mucous secretion was increased by extracts of corpus luteum.

Uterus.—Estrogens cause growth in the capillary bed and thus growth in thickness of the endometrium.[4] This is due partly to cell proliferation and partly to an edema which begins in the deeper portions of the tissue near the myometrium and gradually spreads so that by the time of heat the superficial layers are involved and the cells and connective tissue elements are pushed apart. This edema subsides on the second day.

Estrogens appear to stimulate the deposition of glycogen in uterine epithelium and musculature.[38,39,48,92,135,258,284] However, movement of epithelial glycogen from the basal ends of cells to the luminal ends and its release is apparently influenced by progesterone.[135,291] Lipid is present in

cells of the uterine epithelium during the greater part of diestrus and appears to be under luteal control.[2, 53, 105, 219, 225, 245] Estrogens have also been reported to produce muscular hyperplasia and epithelial proliferation.[87, 111] Both estrone and progesterone have been observed to increase mitoses in uterine epithelium.[52] However, progesterone could only exert its action if the tissues had been previously sensitized by estrone. Corinaldesi[71] reported that cultures of uterine mucosa degenerated unless they contained fragments of granulosa or corpus luteum. Salvatore[260] reported that injection of ovariectomized rats with 100 I.U. of estrone daily for three days caused a doubling of nuclear volume in endometrial cells. Progesterone caused no such change. Asdell *et al.*[5] found that the ratio of cell length to nucleus length was about 4:1 just before heat, reduced to about 2:1 at heat, and continued to decrease for the next two days and then gradually increased throughout the diestrous period. Benoit *et al.*[21] suggested that the activity of gland cells can be measured by estimating the ratio of the area of the nucleus to that of the whole cell. Drasher[85, 86] reported that ribonucleic acid was maximal within the epithelial cells at the time of maximal cytoplasmic function. The cellular ribonucleic acid content was significantly correlated with the water content of the uterus and with the amount of protein nitrogen synthesized. Uterine tissue has been found to be uniformly richer in sodium and poorer in potassium than muscles of the same animals.[324]

Weber *et al.*[317, 318] described the histological nature of postestrous bleeding in cattle. They observed endometrial edema which began during early proestrus and reached a maximum near the time of ovulation. Tissue fluid was discharged from the endometrium in considerable quantity along with the formed elements of blood. Mast cells were observed to accumulate in large numbers in the central pitted areas of caruncles at the onset of metrorrhagia.[320] Larson and Bayley[175] reported that about 20.0 per cent of the cows bred at the time of postestrous bleeding conceived. Trimberger[299] found that the occurrence of postestrous bleeding had no value in predicting whether conception had occurred. Hansel and Asdell[120] observed evidence of microscopic bleeding on the tenth day of the cycle. They suggested that the usual postestrous bleeding is an estrogen-withdrawal phenomenon. Weber *et al.*[319] have also described the occurrence of subserous hemorrhage in the uteri of heifers during the period from estrus to four days postestrus. As far as could be determined, no edema of the perimetrium occurred. Such hemorrhages were not observed in multiparous cows.

Prolonged administration of large doses of estrogens has been shown to produce metaplasia of the uterine epithelium, *i.e.*, conversion of columnar epithelium to stratified squamous.[149, 203, 271, 332] Other changes, such as glandular and endometrial hyperplasia,[44, 73, 331] tumorous growths,[187, 216] and purulent inflammations,[170, 266] have also been observed. Fairly large doses of estrogens have been reported to cause migration of uterine epithelial cell nuclei toward the surface.[228] Large doses of estrogens may also produce metaplasia, cyst formation, or tumor-like papillae in the cervical epithelium.[132, 133]

Oviduct.—Estrogens and gonadotropins have been found to stimulate mucin secretion by epithelial cells of the fallopian tubes of opossums.[210] Weeth[321, 322] reported that the alkaline phosphatase activity in bovine oviducts was increased during the follicular phase of the cycle. The mucosal epithelium showed increased pseudostratification, goblet-like cells, and extruding nuclei during diestrus. Glycogen and small amounts of lipid accumulate during the luteal phase. Murphey[215] also observed extruding cell nuclei in the bovine oviduct. Lombard *et al.*[188] found that mucus-like material was more abundant in the oviduct at three to four days postestrus. Enzmann[91] described "ovoid bodies" which he found in the oviducts of rabbits; he believed them to be formed from cellular debris which acquired a coat of albumin. Henin[126] found that lipids were formed in the oviducts of mice during proestrus and were liberated during estrus. He also described an extrusion from the mucosa of cells of unknown origin; the rate of extrusion was maximal during diestrus and was most frequent near the ostium.

Characteristics of Luminal Fluids

Cervicovaginal Mucus.—According to Masuda *et al.*,[198] the average amount of

vaginal discharge in cows during estrus was 106.8 cc.; the discharge was greatest soon after the beginning of heat. Many reports have been made concerning the measurement of viscosity of cervicovaginal mucus and its interpretation.[66, 106, 107, 221, 249, 268-270] The viscosity is least during estrus and highest during pregnancy. Calisti[56] found that viscosity of vaginal mucus decreased as temperature increased from 25 to 38 C., and that viscosity decreased as the NaCl concentration was increased up to 3.0 per cent. Further increases in NaCl up to 7.0 per cent resulted in increased viscosity of the mucus. He found that pH had no effect on viscosity, nor did diffusing factors in semen (hyaluronidase). Cohen *et al.*[68] found that maximal *"Spinnbarkeit"* was obtained at about the time of ovulation in women. *Spinnbarkeit* was determined by placing a small amount of cervical mucus on a microslide and covering it with a cover slip; the cover slip was then lifted and the maximum length of the mucous thread measured.

When cervical mucus is allowed to dry on a glass slide and is examined microscopically, fernlike crystallization patterns are seen if the mucus is obtained within four to five days before or after estrus.[40, 104, 301, 333] Such arborization is not seen during luteal stages or pregnancy. Appearance of crystallization patterns during the first four months of pregnancy in women has been associated with an increased tendency to abort.[236] Zondek and Rozin[334] reported that dialyzed cervical mucus fails to produce arborization, but that reaction reappears after mixing the dialyzed mucus with electrolytes.

The vaginal fluid of rats has a pH of about 7.2, except during estrus when the pH falls to about 4.4.[19] Estrogens have been found to lower the vaginal pH in monkeys[84] and in women.[265] However, in mares, the vaginal pH during diestrus has been found to be 7.0 to 7.5 and it increased to about 9.0 during estrus.[24, 166] In ewes, the mean vaginal pH during diestrus was 6.69 and fell to 6.6 during estrus.[242] In cows, the usual pH during diestrus is about 6.5 to 7.0 and rises to about 7.6 to 8.1 during heat.[50, 64, 83, 131, 199, 204, 249, 279] In general, the pH of the cervical mucus is about 6.8.[249, 279] Samples tested *in vitro* or from slaughtered animals are usually somewhat more alkaline than when *in vivo* determinations are made.[249] Calisti[55] found the isoelectric point of pregnancy mucus to be about pH 3.0 to 4.0.

In general, the dry matter of cervical mucus averages about 1.2 per cent during estrus, 2.4 per cent during diestrus, and 15.9 per cent during pregnancy.[42, 55, 139, 240, 302, 326] The dry matter of estrous mucus contains about 58.9 per cent ash, 2.9 per cent nitrogen, and 1.5 per cent ether extract, while the dry matter of pregnancy mucus contains about 11.0 per cent ash, 7.7 per cent nitrogen, and 3.0 per cent ether extract. Scott Blair *et al.*[269] reported that nitrogen, dry matter, and viscosity of mucus reached minimum values during estrus.

According to Kaemmerer and Neumann,[146] the reducing substances of cervical mucus in cows vary from 0 to 1,310 mg./100 ml. The addition of hyaluronidase increased the amount of free reducing substances. Boyland,[42] however, reported no effect of hyaluronidase on bovine cervical mucins. Observations of McClean and Rowlands[193] indicate that hyaluronidase is inactive on bovine cervical mucus both during estrus and diestrus. Calisti[56] found no effect of hyaluronidase on the viscosity of vaginal mucus. Boyland[42] found that after hydrolysis about 20.0 to 25.0 per cent of the dry matter of bovine cervical mucus was equivalent to glucose. During diestrus and pregnancy, about 0.5 per cent of the dry matter was N-acetylglucosamine but, during estrus, only traces could be found. Roark and Herman[249] applied iodine tests to bovine mucus and found them positive for glycogen; the biuret test was feebly positive for peptide linkages and the ninhydrin test was negative for free amino acids. The xanthoproteic test indicated the presence of phenyl groups as in tyrosine, tryptophane, and phenylalanine, and a sulfur test indicated the presence of sulfur as in cystine or cysteine. Before acid hydrolysis, 100 ml. of human cervical mucus has been found to contain 50 to 120 mg. of reducing substance and 410 to 790 mg./100 ml. after hydrolysis.[240] Human cervical mucus has been found to contain a polysaccharide consisting of galactose, mannose, fructose, and hexosamine.[22, 46] Cholesterol and phospholipids have also been identified.[47] Werner[323] reported the presence of glycoprotein containing sialic acid as the characteristic sugar component. Supperer[289] found about 833 m.u. of es-

trogen per liter of vaginal mucus from estrous cows.

Uterine Fluid.—According to Amoroso,[3] the term "uterine milk" was first used by Needham in 1667. He also cites references to chemical studies on uterine milk dated in 1842, 1855, and 1864. It was reported that uterine fluid contained about 12.0 per cent dry matter, 0.5 per cent ash, 10.0 per cent protein, 1.0 per cent fat, and no glucose.

The pH of uterine fluids in cows has been reported to be about 5.8 to 7.0[174,-272,273] and varied little during the estrous cycle.[272] However, bacterial infections were generally associated with increasing pH.[273,274] In the mare, uterine pH has been found to be about 6.4 during the luteal phase and 6.9 during estrus.[89] In both rats and mice, the occurrence of estrus was associated with an increase in the pH of uterine fluids.[116] The oxidation reduction potential of rat uterine fluid indicated a strong oxidizing system.[116] Evidence for cyclic variation was inconclusive. The quantity of uterine fluid is relatively large in rats and has been found to play a significant role in transport of spermatozoa and in fertilization.[179,314]

Sturgis[287] studied the flow of uterine fluid in monkeys by means of an uteroabdominal fistula. Adrenalin and intravenous saline were found to increase the rate of flow. Large doses of pilocarpine decreased the flow.

Oviduct Fluid.—A substance positive to Schiff's periodic acid (polysaccharide or glycoprotein, etc.) was found to be secreted by the nonciliated epithelial cells of the oviduct in ewes during proestrus and estrus.[112] The pH of the ewe's oviduct has been found to be 6.0 to 6.4 during diestrus, 6.4 to 6.6 during proestrus, and 6.8 to 7.0 during estrus and metestrus.[113] After specimens had stood for about two hours, the pH fell to 6.2 regardless of the phase of cycle or temperature of refrigeration. The pH of the bovine oviduct has been reported as 6.72.[273]

Follicular Fluid.—The pH of bovine follicular fluid has been reported as about 7.3 to 7.8.[174,192,273] Lutwak-Mann[192] made a chemical analysis of bovine follicular fluid and found it to contain 7.6 per cent dry matter, 252.0 mg./100 ml. Na, 32.0 mg./100 ml. K, 8.6 mg./100 ml. Ca, 1.6 mg./100 ml. Mg, 390.0 mg./100 ml. Cl^-, 5.8 mg./100 ml. inorganic P, 750.0 to 900.0 mg./100 ml. total N, 18.6 mg./100 ml. NPN, 39.0 to 43.0 mg./100 ml. glucose, 13.0 to 95.0 mg./100 ml. lactic acid, and 0.8 to 3.0 mg./100 ml. ascorbic acid. Ross *et al.*[251] found human cystic follicular fluid to contain 0.0226 moles of bicarbonate per liter, 653.0 mg./100 ml. Cl^-, and 67.0 mg./100 ml. glucose. The freezing point of bovine follicular fluid has been reported as —.38 to —.42 C.[137] Human follicular fluid has been found to contain a sulfomucopolysaccharide which is probably responsible for its high viscosity.[222] Bovine follicular fluid has been shown to have antihyaluronidase activity.[220] A glycoprotein has also been identified in follicular fluid.[140]

Bull spermatozoa may live in bovine follicular fluid for as long as 48 hours.[257] However, follicular fluid from pregnant or nonestrous cows caused agglutination and death of the spermatozoa. Kurzrok *et al.*[168] found that human follicular fluid would maintain human spermatozoa for a longer time and in greater activity than seminal plasma. Similarly, Perloff *et al.*[233] found that all samples of human follicular fluid increased sperm motility. The addition of liquor folliculi to uterine mucus and semen *in vitro* prolonged spermatozoal viability.[281] Howeve., Brown[51] and Ross *et al.*[251] reported that sperm motility was not noticeably affected by follicular fluid. Lutwak-Mann[192] examined the effect of follicular fluid on sperm fructolysis and concluded that there was no effect other than that due to the glucose content.

Bovine cystic follicular fluid has been found to contain at least 1,000 r.u. of estrogen per liter.[183] The total amount of estrogenic substance in the liquor folliculi of 1 ton of sow's ovaries has been found to be about 1 mg.[194] D'Amour *et al.*[72] found 950 r.u. of estrogen per liter of sow follicular fluid. Mare follicular fluid has been found to contain about 2 mg. of estrone equivalent per 100 cc.[269] Human follicular fluid has been found to contain 58 to 250 m.u. of estrogen per 100 cc.[233] Follicular fluid has also been found to contain small amounts of progesterone just prior to ovulation.[88,90]

Significance of Luminal Fluids

Sperm Transport.—Spermatozoa have been found to reach the oviduct of rats

within 15 minutes after copulation.[33,34] However, the number of spermatozoa in the oviduct continues to increase for several hours.[35,36] In rabbits, it has been estimated that sperm may reach the upper oviduct less than five minutes after copulation.[165] However, other reports have indicated that the time required is three hours.[43] In women, sperm have been found to reach the oviduct within 30 minutes after their introduction into the vagina.[256] In ewes, some workers found that it took five to eight hours for spermatozoa to reach the oviduct,[74,110,189] while others found spermatozoa in the oviduct in as short a time as six to 30 minutes after insemination.[234,267,285] In cows, the time required for spermatozoa to reach the upper oviduct has been reported as four to five hours,[49] one and three-quarters to four hours,[25] and more recently, less than two to four minutes.[303,305,306]

Parker [229] believes that in rabbits spermatozoa pass through the uterus under their own power but are transported through the oviduct chiefly by means of countercurrents induced by cilia. The effective stroke of the cilia is downward, but the arrangement of folds in the mucosa results in countercurrents toward the ovary. Schilling and Kordts [263] found that on a level plane spermatozoa observed in extirpated surviving uterine tissue migrated in both directions, whereas at an inclination of 30 degrees a few moved upwards, but the majority downwards. At an inclination of 40 degrees, only a few sperm migrated against the strong countercurrent. Rossman [252] observed in rats that uterine peristalses moved semen in circuits from one end of the uterus to the other in a few seconds.

Leonard and Perlman [182] reported that in rats the uterotubal valve permits the passage of living rat spermatozoa at 12 to 72 hours after estrus but will not permit inert particles (India ink) or immobilized rat sperm to be passively carried into the oviduct. Motile bull, mouse, or guinea pig spermatozoa could not enter the rat oviduct. There is evidence that relaxation of the cervix is produced in rats by the presence of the vaginal plug.[31,32] Such relaxation appeared to be necessary for sperm transport.

Asplund [7] described a substance "prostaglandin," in human semen, which had a stimulating effect on rabbit uterine musculature and caused a lowering of tonus in the abdominal tubal ostium. Observation of laparotomized ewes has shown that contractions of longitudinal muscles of the uterus began one to two days before heat, reached a maximum during heat, and gradually ceased after ovulation.[238] Segmental contractions of circular muscle then occurred. Uterine quiescence occurred at about four to five days after heat. Simultaneous contraction of longitudinal muscles and relaxation of circular muscles was thought to assist in opening the cervical canal.

VanDemark and Hays,[124,304] using perfused bovine uteri, found that when semen was deposited midway into the cervix at the same time oxytocin was injected into the perfusate sperm were transported throughout the uterus and into the ovarian portion of the oviduct in a matter of minutes. When no oxytocin was given, either no sperm transport occurred or the extent of travel was limited to the uterus. Yamada [329] described ciliary motion of differing directions in various segments of the oviduct in the frog and tortoise. This was believed to result in countercurrents which caused rotation of the egg and gave it a uniform coating with jelly or shell.

Herman and Horton [131] found that the viscosity of bovine cervical mucus was lowest during the first six hours of estrus and then gradually increased. Berrang [23] found an inverse relationship between the viscosity of mucus and the penetrability by spermatozoa. Barton and Wiesner [18] showed that semen and cervical mucus were virtually immiscible and that fertility depended on the ability of sperm to penetrate the interface between mucus and semen. However, Wunsche [328] observed that sperm did not penetrate the mucus but traveled along its surface. Lamar *et al.*[172] found that human cervical mucus rendered the cervix temporarily penetrable by spermatozoa for a period of about four days between days 9 and 19 of the cycle.

Khreninger-Guggenberger [157] observed that when a pipette filled with physiological saline solution was placed vertically into a dish of semen, the sperm did not migrate upward into the pipette. However, when the saline was replaced by starch or dextrin solution, the sperm did enter. Likewise, when the cervix of a freshly ex-

tirpated uterus was placed in contact with semen, no sperm were found in the uterine body within 12 hours unless mucus was expressed so that it came in contact with the semen. Abarbanel [1] reported that, in women just before ovulation, cervical mucus increased in volume, decreased in viscosity, showed a decrease or total absence of leukocytes, and was temporarily penetrable by spermatozoa. These changes were shown to be due to the action of estrogens.

Raps [243] reported that the amount of mucus present in the genital tracts of cows at the time of insemination had no significant effect on conception rate. DeVuyst and Vervaek [80] found no relationship between the degree of viscosity of cervical mucus and the conception rate. However, Filatova [95] found that increasing elasticity of vaginal mucus was related to increasing conception rate.

The addition of "mucinase" (hyaluronidase) to diluted bull semen has been reported to increase fertility by 18.0 to 20.0 per cent.[6, 282, 283] These authors believed that the effect was probably due to a reduction in viscosity of mucus. However, as previously mentioned, it appears that hyaluronidase does not affect the viscosity of mucus. Calisti and Quacquarini [57] reported that the addition of hyaluronidase to high dilutions of bull semen had no beneficial action on fertility. Chang [63] found that the application of hyaluronidase to the vagina before insemination or the addition of hyaluronidase to the sperm suspension had no significant effect on the fertilization of rabbit ova *in vivo*. Fiorentino and Caretta [96] obtained inconclusive results from the addition of hyaluronidase to bull semen; 3 bulls showed slight increases in fertility, while 1 bull showed a decrease. Johnston and Mixner [145] found no significant correlation between the fertility of bull semen and its hyaluronidase content. Sallman and Birkeland [259] obtained evidence that high concentrations of hyaluronidase in semen were, in fact, associated with decreased fertility.

Viability of Spermatozoa.—The maximum duration of fertilizing capacity of spermatozoa in the female genital organs of rats has been reported as 14 hours.[280] In mice, spermatozoa have been found to enter the oviduct within one and one-fourth hours; fertilizing ability was retained for about six hours in the oviduct, although motility lasted 13.5 hours.[205] In guinea pigs, fertilizing power was retained in the female genital tract for 22 hours, but a decrease in the percentage of fertile inseminations occurred after 17 hours.[281] In rabbits, sperm have been found to retain their fertility in the female tract up to 30 hours.[117] In women, spermatozoa have been reported to retain fertility for 40 to 80 hours.[60, 67, 144, 256, 286, 316] Viability has been reported as one hour in the vagina, 25 hours in the uterus, and 40 hours in the cervix.[144] In ewes, spermatozoa have been reported to remain viable about six to 20 hours in the vagina, 24 to 80 hours in the cervix, nine to 12 hours in the uterus, and only a few hours in the oviduct.[75, 109, 169, 239, 241] The duration of spermatozoan fertility in mares has been reported as 28 hours to six days.[54, 77, 217, 231] In cattle, it has been reported that sperm may remain fertile in the female genital tract for 15 to 56 hours.[25, 161, 171, 307, 308] Beshlebnov [25] found that survival of sperm was longest in the cervix (24 to 30 hours), whereas sperm in the vagina were immobilized after four hours, while in the uterus and tubes degeneration occurred after ten to 15 hours.

Fertilization.—Austin [14, 15] and Austin and Braden [16, 17] reported that rat sperm required about four hours in the female tract before they are capable of fertilizing eggs. Chang [62, 63] made similar observations in rabbits. He found that ejaculated or epididymal rabbit spermatozoa required at least six hours' stay in the oviducts to acquire fertilizing capacity. The development of fertilizing capacity of sperm could be partially achieved in the uterus and then completed in the tubes. Zittle and O'Dell [330] reported evidence for a keratin-like protein, perhaps on the surface, of bull sperm. Green [108] found that the membrane of ram sperm was insoluble in many protein solvents and suggested that it was probably an albuminoid. It may be speculated that "capacitation" involves a chemical action on the sperm membrane preparatory to dissolution. Such dissolution is undoubtedly necessary before fusion of male and female pronuclei can occur. Blandau and Odor [37] found that the external sheaths of the middle and main piece gradually disappeared nine to 19

hours after the sperm penetrated the ooplasm.

Moricard [211] suggested that follicular fluid may have a meiogenic action on ova. The same author [212] observed that no spermatozoa penetrated the zona pellucida of the rabbit oocyte when the latter was cultivated *in vitro* under aerobic conditions. However, under relatively anaerobic conditions, spermatozoa penetrated the zona pellucida of 14 out of 21 oocytes.

Swyer [291] observed that artificially ovulated rabbit eggs were denuded of cumulus cells by insertion for 20 to 30 minutes in a hyaluronidase solution, but the corona radiata remained intact. A fallopian tube extract had no such effect but, when eggs denuded of cumulus cells were inserted into the fallopian tubes of normal pseudopregnant rabbits, the corona radiata was removed within three hours. Smith [278] found that when small pieces of fallopian tube were added to egg cultures, some corona cells dropped off but, more important, parthenogenetic cleavage and fragmentation were almost completely prevented.

Resistance to Infection.—It has been well established that the uteri of estrous rabbits are more resistant to infections than those of pseudopregnant rabbits.[27-29, 201] Likewise, the bovine uterus has been found to be more resistant to infection during estrus than during diestrus.[30, 173, 254, 255] This phenomenon has been attributed to the action of progesterone in reducing resistance, since untreated ovariectomized animals had resistance similar to estrous animals, while progesterone-treated animals were more subject to infection.[29] However, there is some evidence that estrogen treatment of ovariectomized cows has a positive influence in increasing resistance.[254, 255] Older cows and repeat breeders have been observed to have more resistance than heifers or first-service cows.[27, 30] Lundgren [191] reported the successful use of stilbestrol in treating purulent endometritis. Brauchhardt [45] reported that estrogens produced a 60.0 per cent increase in the weight of lymph nodes in guinea pigs. Also, it has been reported that estrus in cows is associated with an increase in total leukocytes as well as in the relative number of polymorphonuclear leukocytes.[85, 93] Hawk *et al.*[123] injected *Escherichia coli* suspensions into the peritoneal cavities of estrous and pseudopregnant rabbits and no difference was found in the number of bacteria recovered from the peritoneal cavities of the two types of rabbits 28 hours later. However, the numbers recovered from the uteri differed considerably, being higher in the pseudopregnant rabbits.

Agglutinins against *Trichomonas foetus* have been demonstrated in both uterine and vaginal fluids of previously infected cows.[154, 235] Likewise, agglutinins for Brucella [142] and *Vibrio fetus* [41, 138, 176, 202, 237, 248] have been demonstrated. In the case of *V. fetus*, the agglutinins of cervicovaginal mucus were observed to persist longer than blood serum antibodies. Lawson and MacKinnon [176] reported that among cows infected with *V. fetus*, positive cultures were more readily obtained from mucus during estrus, whereas positive agglutination tests were more frequent at other stages of the cycle. Hughes [138] found that samples obtained by the tampon method were more likely to be positive than were samples of aspirated mucus. Boyd [41] reported that positive reactions were less frequent during the last three months of pregnancy.

Leonard and Kuzrok [181] immunized rats against bull testis extracts and found that uterine fluid from an immunized rat inhibited the action of hyaluronidase, whereas uterine fluid of control rats did not. However, follicle cells of ova from immunized rats were dispersed as readily as those from normal rats.

Young calves, injected with gonad-stimulating hormones, developed serum antibodies when exposed to *Brucella abortus*, whereas untreated control calves failed to develop such antibodies.[315]

Verna *et al.*[311] isolated about 50 different microorganisms from the vaginas of cows. Using cellular suspensions of these organisms as antigen, agglutination against serums of cows was observed.

Spermatozoa have been observed to possess specific antigenic properties.[82, 127, 129, 276, 277] Using radioactive P^{32}, it has been found that sperm P was rapidly absorbed from the female genital tract.[20] However, attempts to induce sterility by active immunization against spermatozoa have been unsuccessful.[128, 130, 230] Kato [148] described agglutination of rabbit sperm by vaginal and uterine secretions both *in vivo* and *in vitro*. However, he believed the action to

be due to neutral salts contained in the secretions. Lindahl and Kihlstrom [185] reported the presence of an antiagglutinating factor in seminal plasma of man and 4 other mammals. The substance is reported as being a protein containing a carbohydrate group and its activity is restricted to the reduced form since oxidation causes it to lose its ability of being fixed to the cell surface. Lindahl and Nilsson [186] observed an antagglutin action in follicles and oviducts of rabbits following ovarian stimulation, either during estrus, by hormone injections, or by mating. This antagglutin was also found in the follicles of cattle and swine. Its action was not species specific. Kibrich *et al.*[158,159] described procedures for detecting antibodies against spermatozoa.

CONCLUSION

Unfortunately, a knowledge of the subject matter in this review contributes little to one's specific ability to diagnose and treat infertility. However, it seems clear that research workers are slowly but surely placing physiology on a foundation of cellular biochemistry. There is adequate evidence that proper functioning of the reproductive organs is dependent upon many intricate factors involving the circulatory, nervous, and endocrine systems. Correlating the chemical and physical characteristics of genital tract fluids with histochemical and endocrinological activity remains a challenge, but offers hope in understanding problems of fertility. Superimposed on these physiological mechanisms, of course, are a host of bacteriological and pathological processes, all of which must be properly assessed in the diagnosis and treatment of infertility.

References

[1] Abarbanel, A. R.: Spermatozoa and Cervical Mucus, in "The Problem of Fertility." E. T. Engle (ed.), Princeton University Press, Princeton, N.J., 1946.

[2] Alden, R. H.: Further Studies on the Alteration of Epithelial Fat in the Rat Uterus During the Estrous Cycle, Early Pregnancy, and in the Treated and Untreated Castrate. Anat. Rec., *94*, (1946): 445–446.

[3] Amoroso, E. C.: Placentation, in "Marshall's Physiology of Reproduction." A. S. Parkes (ed.), Longmans, Green and Co., London, 1952.

[4] Asdell, S. A.: Cattle Fertility and Sterility. Little, Brown and Co., Boston, 1955.

[5] Asdell, S. A., de Alba, J., and Roberts, S. J.: Studies on the Estrous Cycle of Dairy Cattle: Cycle Length, Size of Corpus Luteum, and Endometrial Changes. Cornell Vet., *39*, (1949): 389–402.

[6] Aslanjan, M. M.: The Effectiveness of the Use of Mucinase in the Artificial Insemination of Cows. Sovet. Zootech., *8*, (1953): 86–88 (Anim. Breed. Abstr., *21*, [1953]: 351).

[7] Asplund, J.: Some Preliminary Experiments in Connection with the Effect of Prostaglandin on the Uterus and Tubae *in Vivo*. Acta Physiol. Scand., *13*, (1947): 109–114 (Anim. Breed. Abstr., *17*, [1949]: 83).

[8] Ascher, A. W., and Turner, C. J.: Vaginal Sulfydryl and Disulfide Groups During the Estrous Cycle of the Mouse. Nature, *175*, (1955): 900–901.

[9] Astwood, E. B.: A Six-Hour Assay for the Quantitative Determination of Estrogen. Endocrinology, *23*, (1938): 25–31.

[10] Astwood, E. B.: Time Relationship in the Growth and Water Exchange of the Uterus Following Estrogenic Stimulation. Anat. Rec., *70*, (1938, suppl. 3): 5.

[11] Astwood, E. B.: Changes in the Weight and Water Content of the Uterus of the Normal Adult Rat. Am. J. Physiol., *126*, (1939): 162–170.

[12] Astwood, E. B.: Factors Influencing the Early Action of Estrogen upon the Uterus. Am. J. Physiol., *129*, (1940): 302–303.

[13] Atkinson, W. B., and Elftman, H.: Effect of Steroid Sex Hormones on Distribution of Alkaline Phosphatase in Uterus of the Mouse. Proc. Soc. Exptl. Biol. and Med., *62*, (1946): 148–150.

[14] Austin, C. R.: Observations on the Penetration of the Sperm into the Mammalian Egg. Austral. J. Scient. Res., *B 4*, (1951): 581–596.

[15] Austin, C. R.: The "Capacitation" of the Mammalian Sperm. Nature, *170*, (1952): 626.

[16] Austin, C. R., and Braden, A. W. H.: Passage of the Sperm and the Penetration of the Egg in Mammals. Nature, *170*, (1952): 919–921.

[17] Austin, C. R., and Braden, A. W. H.: Time Relations and Their Significance in the Ovulation and Penetration of Eggs in Rats and Rabbits. Austral. J. Biol. Sci., *7*, (1954): 179–195.

[18] Barton, M., and Wiesner, B. P.: The Receptivity of Cervical Mucus to Spermatozoa. Brit. M. J., *2*, (1946): 606–610.

[19] Beilly, J. S.: Hydrogen Ion Concentration Changes in the Vaginal Fluid of the Rat During an Estrous Cycle. Endocrinology, *23*, (1939): 275–277.

[20] Bengtsson, L.: Sperm Absorption in Rabbits. Anim. Breed. Abstr., *19*, (1951): 510.

[21] Benoit, J., Kehl, R., and Leportois, M.: Biometric Techniques Applied to the Histophysiology of Glandular Organs, Particularly in the Field of Endocrinology. Cytometric Techniques. Histometric Techniques. Compt. rend. Soc. de biol., *136*, (1942): 514–519 (Anim. Breed. Abstr., *12*, [1944]: 49).

[22] Bergman, P., and Werner, I.: Analysis of Carbohydrates in Human Cervical Mucus. Acta obst. et gynec. Scand., *30*, (1951): 273–277 (abstr. in Fert. and Ster., *3*, [1952]: 88).

[23] Berrang, K.: Mucus Penetrating Power of Bull Spermatozoa *in Vitro*. Anim. Breed. Abstr., *21*, (1953): 47.

[24] Berthelon, M., and Juteau, J.: Variations in Vaginal pH During the Estrous Cycle in the Mare. Compt. rend. Soc. de Biol., *139*, (1945): 459–460 (Anim. Breed. Abstr., *14*, [1946]: 138).

[25] Beshlebnov, A. V.: The Optimal Time of Inseminating Cows During Estrus. Probl. Zivotn., *2*, (1938): 73–88 (Anim. Breed. Abstr., *6*, [1938]: 291–292).

[26] Beyler, A. L., and Stegg, C. M.: Endocrine Regulation of *Beta*-Glucuronidase Activity. Am. J. Physiol., *167*, (1951): 768.

[27] Black, W. G., Simon, J., Kidder, H. E., and Wiltbank, J. N.: Bactericidal Activity of the Uterus in the Rabbit and the Cow. Am. J. Vet. Res., *15*, (1954): 247–251.

[28] Black, W. G., Simon, J., McNutt, S. H., and Casida, L. E.: Bactericidal Activity of the Uterus in Different Endocrine States. Abstr. in J. Dai. Sci., *36*, (1953): 586–587.

[29] Black, W. G., Simon, J., McNutt, S. H., and Casida, L. E.: Investigations on the Physiological Basis for the Differential Response of Estrous and Pseudopregnant Rabbit Uteri to Induced Infection. Am. J. Vet. Res., *14*, (1953): 318–323.

[30] Black, W. G., Ulberg, L. C., Kidder, H. E., Simon, J., McNutt, S. H., and Casida, L. E.: Inflammatory

Response of the Bovine Endometrium. Am. J. Vet. Res., *14*, (1953): 179–183.

[31] Blandau, R. J.: Is the Copulation Plug Essential for the En Masse Transport of Spermatozoa into the Uterine Cornua of the Albino Rat? Anat. Rec., *91*, (1945): 266–267.

[32] Blandau, R. J.: On the Factors Involved in Sperm Transport Through the Cervix Uteri of the Albino Rat. Am. J. Anat., *77*, (1945): 253–272.

[33] Blandau, R. J., Jordan, E. S., Money, W. L., and Ring, J. R.: The Rate of Transport of Spermatozoa in the Fallopian Tubes of the Rat. Anat. Rec., *81*, (1941): 83.

[34] Blandau, R. J., and Money, W. L.: Observations on the Rate of Transport of Spermatozoa in the Female Genital Tract of the Rat. Anat. Rec., *90*, (1944): 255–260.

[35] Blandau, R. J., and Odor, D. L.: The Number of Spermatozoa Reaching Various Segments of the Oviduct of the Rat, 12, 24 and 36 Hours After Mating. Anat. Rec., *100*, (1948): 733.

[36] Blandau, R. J., and Odor, D. L.: The Total Number of Spermatozoa Reaching Various Segments of the Reproductive Tract in the Female Albino Rat at Intervals After Insemination. Anat. Rec., *103*, (1949): 93–109.

[37] Blandau, R. J., and Odor, D. L.: Observations on Sperm Penetration into the Ooplasm and Changes in the Cytoplasmic Components of the Fertilizing Spermatozoon in Rat Ova. Fert. and Ster., *3*, (1952): 13–26.

[38] Bo, W. J., and Atkinson, W. B.: Histochemical Studies on Glycogen Deposition in Uterus of the Rat. III. Effect of Starvation. Proc. Soc. Exptl. Biol. and Med., *83*, (1953): 405–407.

[39] Boettiger, E. G.: Changes in the Glycogen and Water Content of the Rat Uterus. J. Cell. and Comp. Physiol., *27*, (1946): 9–14.

[40] Bone, J. F.: Crystallization Patterns in Vaginal and Cervical Mucus Smears as Related to Bovine Ovarian Activity and Pregnancy. Am. J. Vet. Res., *15*, (1954): 542–547.

[41] Boyd, H.: Bovine Genital Vibriosis. Bull. Roy. Vet. Coll., Stockholm, 1955.

[42] Boyland, E.: The Composition of Bovine Cervical Mucins and Their Reactions with Oxidizing Agents. Biochem. J., *40*, (1946): 334–337.

[43] Braden, A. W. H.: Distribution of Sperm in the Genital Tract of the Female Rabbit After Coitus. Austral. J. Biol. Sci., *6*, (1953): 693–705.

[44] Braden, A. W. H., and Paterson, J. E.: The Persistence of Endometrial Cysts Induced by Oestrogen in Guinea Pigs. Austral. J. Biol. Sci., *6*, (1953): 520–531.

[45] Brauchhardt, G.: The Influence of the Sex Hormones on the Size of the Lymph Nodes and on Mitosis. Ztschr. Vitaminforsch., Hormon-u. Fermentforsch., *5*, (1953): 373–380 (Biol. Abstr., *29*, [1955]: 313).

[46] Brechenridge, M. A. B., and Pommerenke, W. T.: Analysis of Carbohydrates in Human Cervical Mucus. Fert. and Ster., *2*, (1951): 29–44.

[47] Brechenridge, M. A. B., and Pommerenke, W. T.: Analysis of Lipid Constituents of the Cervical Secretion. Fert. and Ster., *2*, (1951): 451–458.

[48] Breipohl, W., and Schmitz, A.: Studies on the Relative Weight and Glycogen Content of the Ovary, Uterus and Testis in Slaughtered Animals. Ztschr. f. Geburtsh. u. Gynäk., *108*, (1934): 330–338 (Anim. Breed. Abstr., *4*, [1936]: 151).

[49] Brewster, J. E., May, R., and Cole, C. L.: The Time of Ovulation and Rate of Spermatozoa Travel in Cattle. Proc. Am. Soc. Anim. Prod. (1940): 304–311.

[50] Brown, P. C.: Physiological and Histological Changes in the Vagina of the Cow During the Estrual Cycle. Am. J. Vet. Res., *5*, (1944): 99–112.

[51] Brown, R. L.: Effect on Spermatozoa of Tissue Fluids Encountered in the Female Genital Tract. Am. J. Obst. and Gynec., *46*, (1943): 873–876.

[52] Bureau, V.: Action of Estrone and Progesterone on the Uterine Horn of Rabbits, Studied by the Colchicine Technique. Compt. rend. Soc. de biol., *130*, (1939): 933–936 (Anim. Breed. Abstr., *8*, [1940]: 186).

[53] Bulliard, H., and Grundland, I.: Development of Fats in the Mucus of the Genital Tract. Compt. rend. Soc. de biol., *140*, (1946): 259–261 (Anim. Breed. Abstr., *16*, [1948]: 349).

[54] Burkhardt, John: Sperm Survival in the Genital Tract of the Mare. J. Agric. Sci., *39*, (1949): 201–203.

[55] Calisti, V.: The Isoelectric Point of the Mucus from the Cow's Genital Tract. Clin. Vet., *69*, (1946): 97–105 (Anim. Breed. Abstr., *16*, [1948]: 121).

[56] Calisti, V.: The Viscosity of the Mucus of the Genital Tract of the Cow in Relation to Temperature, Salts, pH and Diffusing Factors. Zootec. e Vet., *3*, (1948): 131–138 (Anim. Breed. Abstr., *16*, [1948]: 221).

[57] Calisti, V., and Quacquarini, P.: The Fertility of Bull Semen Treated with Hyaluronidase. Anim. Breed. Abstr., *21*, (1953): 48.

[58] Carroll, W. F.: Influence of Estrogen on Respiration of Rat Uterine Tissue. Proc. Soc. Exptl. Biol. and Med., *49*, (1942): 50–52.

[59] Carroll, W. R.: Variations in the Water Content of the Rat's Uterus During Continuous Estrogenic Treatment. Endocrinology, *36*, (1945): 266–271.

[60] Cary, W. H.: Duration of Sperm Cell Migration in Uterine Secretions. J. Am. M. A., *106*, (1936): 2221–2223.

[61] Cesa, L.: The action of Folliculin and of the Corpus Luteum upon the Glands of the Cervix. Compt. rend. Soc. de biol., *122*, (1936): 1237–1238 (Anim. Breed. Abstr., *5*, [1937]: 472).

[62] Chang, M. C.: Fertilizing Capacity of Spermatozoa Deposited into the Fallopian Tubes. Nature, *168*, (1951): 697–698.

[63] Chang, M. C.: Development of Fertilizing Capacity of Rabbit Spermatozoa in the Uterus. Nature, *175*, (1955): 1035–1036.

[64] Choudhuri, A. C., and Prasad, R. B.: A Study of the Reaction of the Vaginal Secretion in Cows and Heifers. Indian J. Vet. Sci. and Anim. Husb., *24*, (1954): 81–87.

[65] Christian, R. E., Dreher, W. H., and Casida, L. E.: Additional Observations on the Nature of Reproductive Failures in Cows of Low Fertility. J. Anim. Sci., *8*, (1949): 638–639.

[66] Clift, A. F., and Hart, J.: Variations in the Apparent Viscosity of Human Cervical Mucus. J. Physiol., *122*, (1953): 358–365.

[67] Cohen, M. R., and Stein, I. F.: Sperm Survival at Estimated Ovulation Time. Comparative Morphology: Relative Male Infertility. Fert. and Ster., *2*, (1951): 20–27.

[68] Cohen, M. R., Stein, I. F., and Kay, B. M.: Spinnbarkeit: A Characteristic of Cervical Mucus. Fert. and Ster., *3*, (1952): 201–209.

[69] Cole, D. F.: The Effects of Oestradiol on the Rat Uterus. J. Endocrinol., *7*, (1950): 12–23.

[70] Cole, H. H.: A Study of the Mucosa of the Genital Tract of the Cow, with Special Reference to the Cyclic Changes. Am. J. Anat., *46*, (1930): 261–301.

[71] Corinaldesi, F.: Influence of Granulosa and Corpus Luteum upon Tissue Culture of Uterine Mucosa. Boll. soc. Ital. Biol. e Sper., *9*, (1934): 719–721 (Anim. Breed. Abstr., *2*, [1934]: 382).

[72] D'Amour, F. E., D'Amour, M. C., and Gustavson, R. G.: The Estrin Content of Sow Ovaries. J. Pharmacol. and Exptl. Therap., *49*, (1933): 141–145.

[73] Dangoumau, L. M. R.: Atypical Endometria and Corresponding Ovaries. A Histological Study. Arch. d'anat., d'histol. et d'embryol., *36*, (1953): 1–60 (Biol. Abstr., *29*, [1955]: 1601).

[74] Dauzier, L., and Wintenberger, S.: Mammalian Fertilization: Spermatozoal Transport in the Genital Tract of the Ewe. Compt. rend. Soc. de biol., *146*, (1952): 67–70 (Anim. Breed. Abstr., *20*, [1952]: 358).

[75] Dauzier, L., and Wintenberger, S.: Mammalian Fertilization. I. Duration of the Fertilizing Ability of Ram Spermatozoa in the Genital Tract of the Ewe, and Duration of Fertility of the Ovum After Ovulation. II. The Transport of Spermatozoa in the Genital Tract of the Dioestrous and Anoestrus Ewe. Compt. rend. Soc. de biol., *146*, (1952): 660–665 (Anim. Breed Abstr., *21*, [1953]: 58).

[76] David, J. C.: The Action of Oestrin on the Oxygen Consumption of the Uteri of Mice. J. Pharmacol. and Exptl. Therap., *43*, (1931): 1–11.

[77] Day, F. T.: Survival of Spermatozoa in the Genital Tract of the Mare. J. Agric. Sci., *32*, (1942): 108–111.

[78] Demmel, M.: Blood Sugar in the Sexual Cycle of Cattle. Anim. Breed. Abstr., *1*, (1933): 9.

[79] Desclin, L.: Vaginal Mucification in the Guinea-Pig. Compt. rend. Soc. de biol., *115*, (1934): 439–441 (Anim. Breed. Abstr., *3*, [1934]: 70).

[80] DeVuyst, A., and Vervack, W.: The Relation Between the Viscosity of the Cervical Mucus in the Cow and Fertility. Ann. Med. Vet., *94*, (1950): 298–300 (Anim. Breed. Abstr., *18*, [1950]: 397).

[81] Dirscherl, W., and Breuer, H.: The Effect of Male and Female Sex Hormones on Respiration, Aerobic and Anaerobic Glycolysis of Mouse Uterus. Biochem. Ztschr., *324*, (1953): 41–50 (Biol. Abstr., *28*, [1954]: 2005).

[82] Docton, F. L., Ferguson, L. C., Lazear, E. J., and Ely, F.: The Antigenicity of Bovine Spermatozoa. J. Dai. Sci., *35*, (1952): 706–709.

[83] Dougherty, R. W.: *In Vivo* Determinations of the Hydrogen Ion Concentration of the Vagina of Dairy Cows. North Am. Vet., *22*, (1941): 216–219.

[84] Dow, D., and Zuckerman, S.: Further Observations on the Vaginal pH of Monkeys Treated with Estrogenic Substances. Endocrinology, *25*, (1939): 525–528.

[85] Drasher, M. L.: Morphological and Chemical Observations on the Mouse Uterus During the Estrous Cycle, and Under Hormonal Treatment. J. Exptl. Zool., *119*, (1952): 333–353.

[86] Drasher, M. L.: Aging Changes in Nucleic Acid and Protein-Forming Systems of the Virgin Mouse Uterus. Proc. Soc. Exptl. Biol. and Med., *84*, (1953): 596–601.

[87] Dux, K., and Soszka, S.: Histological Studies on the Sexual Cycle of the Guinea Pig. Arch. de biol., *51*, (1940): 1–47 (Anim. Breed. Abstr., *9*, [1941]: 268).

[88] Duyvene de Wit, J. J.: The Occurrence of Progesterone and 17-Ketosteroids in Liquor Folliculi of Pigs, Cattle and Women. Acta brev. Neerland., *12*, (1942): 23–25 (Anim. Breed. Abstr., *15*, [1947]: 85).

[89] Dybing, O.: Preliminary Observations on the Acidity of the Genital Tract in the Mare. Maanedsskr. Dyrl., *47*, (1935): 561–569 (Anim. Breed. Abstr., *5*, [1937]: 260).

[90] Edgar, D. G.: Progesterone in Body Fluids. Nature, *170*, (1952): 543–544 (Anim. Breed. Abstr., *21*, [1953]: 19).

[91] Enzmann, E. V.: "Ovoid Bodies" from the Oviducts of Rabbits. Anat. Rec., *71*, (1938): 125–131.

[92] Ericksen, S.: Histochemical Changes in the Endometrium of the Dog During the Oestrous Cycle. Acta path. et microbiol. Scand., *33*, (1953): 263–267 (Biol. Abstr., *28*, [1954]: 2813).

[93] Feja, E.: Investigations on the Behavior of the Blood During Natural Estrus and Estrus Induced by "Merck Sexocretin" in Cattle. Dissertation, Berlin, 1941 (Anim. Breed. Abstr., *11*, [1943]: 22).

[94] Ferring, N. L.: Vaginal Smears and Estrus Diagnosis in Cattle. Dissertation, Munich, 1937 (Anim. Breed. Abstr., *8*, [1940]: 367).

[95] Filatova, K. N.: Fertilization of Cows in Relation to the State of Oestrus. Sovet. Zootech., *7*, (1952): 90–99 (Anim. Breed. Abstr., *20*, [1952]: 231).

[96] Fiorentino, A., and Caretta, A.: The Results Obtained from the Addition of Crystalline Hyaluronidase to the Semen of *B. Taurus*. Anim. Breed. Abstr., *19*, (1951): 465.

[97] Fishman, W. H.: β-Glucuronidase: Its Relation to the Action of the Estrogenic Hormones. J. Biol. Chem., *169*, (1947): 7–15.

[98] Fishman, W. H., and Fishman, L. W.: The Elevation of Uterine β-Glucuronidase Activity by Estrogenic Hormones. J. Biol. Chem., *153*, (1944): 487–488.

[99] Foley, R. C., and Reece, R. P.: Histological Studies of the Bovine Uterus, Placenta and Corpus Luteum. Massachusetts Agric. Exper. Sta. Bull. 468, 1953.

[100] Foley, R. C., Reece, R. P., and Leathem, J. H.: Histochemical Observations of the Bovine Uterus, Placenta and Corpus Luteum During Early Pregnancy. J. Anim. Sci., *13*, (1954): 131–137.

[101] Ford, D. H.: The Role of Progesterone in the Production of Vaginal Changes in Ovariectomized Female Rats. Endocrinology, *55*, (1954): 230–231.

[102] Fuenzolida, F.: On the Influence of 3-Keto-Steroids on the Phosphatase Content of Atypically Proliferating Uterine Epithelium in the Guinea Pig. Endocrinology, *45*, (1949): 231–241 (Anim. Breed. Abstr., *18*, [1950]: 83).

[103] Fulton, J. F.: A Textbook of Physiology. 17th ed. W. B. Saunders Co., Philadelphia, 1955.

[104] Garm, O., and Skjerven, O.: Studies on Cervical Mucus for Early Diagnosis of Pregnancy and Endocrine Changes in the Reproductive Cycle in Domestic Animals. Nord. Vet.-med., *4*, (1952): 1098–1103 (Biol. Abstr., *27*, [1953]: 8–9).

[105] Gilbert, C.: The Distribution of Fat in the Endometrium of the Castrate Rabbit After Treatment with Estradiol Benzoate and Progesterone. Endocrinology, *30*, (1942): 773–781.

[106] Glover, F. A., and Scott Blair, G. W.: Bovine Sterility and Pregnancy in Relation to the Rheological Properties of Cervical Secretions. Nature, *167*, (1951): 285.

[107] Glover, F. A., and Scott Blair, G. W.: The Flow Properties of Cervical Secretions in the Cow as Related to Certain Physiological Conditions. J. Endocrinol., *9*, (1953): 160–169.

[108] Green, W. W.: The Chemistry and Cytology of the Sperm Membrane of Sheep. Anat. Rec., *76*, (1940): 455–473.

[109] Green, W. W.: Duration of Sperm Fertility in the Ewe. Am. J. Vet. Res., *8*, (1947): 299–300.

[110] Green, W. W., and Winters, L. M.: Studies on the Physiology of Reproduction in the Sheep. III. The Time of Ovulation and Rate of Sperm Travel. Anat. Rec., *61*, (1935): 457–469.

[111] Grumbrecht, P., and Laeser, A.: The Local Action of Follicular Hormone on Uterine Mucosa. Klin. Wchnschr., *17*, (1938): 1086–1088 (Anim. Breed. Abstr., *6*, [1938]: 352).

[112] Hadek, R.: Mucin Secretion in the Ewe's Oviduct. Nature, *171*, (1953): 750 (Anim. Breed. Abstr., *21*, [1953]: 274).

[113] Hadek, R.: Alternation of pH in the Sheep's Oviduct. Nature, *171*, (1953): 976 (Anim. Breed. Abstr., *21*, [1953]: 274).

[114] Hagerman, D. D., and Villee, C. A.: Effects of Estradiol on the Metabolism of Human Endometrium *in Vitro*. J. Biol. Chem., *203*, (1953): 425–431.

[115] Hagerman, D. D., and Villee, C. A.: Effects of the Menstrual Cycle on the Metabolism of Human Endometrium. Endocrinology, *53*, (1953): 667–673.

[116] Hall, B. V.: Variations in Acidity and Oxidation Reduction Potential in Rodent Uterine Fluids. Physiol. Zool., *9*, (1936): 471–497.

[117] Hammond, J., and Asdell, S. A.: The Vitality of the Spermatozoa in the Male and Female Reproductive Tracts. J. Exptl. Biol., *4*, (1926): 155–185.

[118] Hansel, Wm.: The Effects of Estrogen and Progesterone on the Arterial System of the Uterus of the Cow. J. Dai. Sci., *33*, (1950): 381.

[119] Hansel, Wm., and Asdell, S. A.: The Effects of Estrogen and Progesterone on the Arterial System of the Uterus of the Cow. J. Dai. Sci., *34*, (1951): 37–44.

[120] Hansel, W., and Asdell, S. A.: The Causes of Bovine Metestrous Bleeding. J. Anim. Sci., *11*, (1952): 346–354.

[121] Hansel, W., Asdell, S. A., and Roberts, S. J.: The Vaginal Smear of the Cow and the Cause of Its Variation. Am. J. Vet. Res., *10*, (1949): 221–228.

[122] Harris, R. S., and Cohen, S. L.: The Influence of Ovarian Hormones on the Enzymatic Activities of Tissues. Endocrinology, *48*, (1951): 264–272.

[123] Hawk, H. W., Simon, J., Cohen, H., McNutt, S. H., and Casida, L. E.: The Relative Bactericidal Activity of the Uterine and Body Cavities of Estrous and Pseudopregnant Rabbits. J.A.V.M.A., *126*, (1955): 268–270.

[124] Hays, R. L., and VanDemark, N. L.: Effect of Hormones on Uterine Motility and Sperm Transport in

the Perfused Genital Tract of the Cow. (abstr.) J. Dai. Sci., *35*, (1952): 499–500.

[135] Hechter, O., Krohn, L., and Harris, J.: Effects of Estrogens and Other Steroids on Capillary Permeability. Endocrinology, *30*, (1942): 598–608.

[136] Henin, A.: Study of the Modifications of the Oviduct of the Mouse During the Estrous Cycle. Arch. de biol., *52*, (1941): 97–115 (Anim. Breed. Abstr., *12*, [1944]: 153).

[137] Henle, W.: The Specificity of Some Mammalian Spermatozoa. J. Immunol., *34*, (1938): 325–336 (Anim. Breed. Abstr., *7*, [1939]: 374).

[138] Henle, W., and Henle, G.: Spermatozoal Antibodies and Fertility. II. Attempt to Induce Temporary Sterility in Female Guinea Pigs by Active Immunization Against Spermatozoa. J. Immunol., *38*, (1940): 105–115.

[139] Henle, W., Henle, G., and Chambers, L. A.: Studies on the Antigenic Structure of Some Mammalian Spermatozoa. J. Exptl. Med., *68*, (1938): 335–352 (Anim. Breed. Abstr., *7*, [1939]: 178–179).

[140] Henle, W., Henle, G., Church, C. F., and Foster, C.: Spermatozoal Antibodies and Fertility. I. Attempt to Induce Temporary Sterility in Female White Mice by Passive Immunization with Spermatozoal Antisera. J. Immunol., *38*, (1940): 97–103.

[141] Herman, H. A., and Horton, O. H.: Spermatozoal Behavior in Bovine Cervical Mucus at Varying Stages of Estrus. J. Dai. Sci., *31*, (1948): 679–680.

[142] Herold, L., and Effkemann, G.: On Epithelial Metaplasia of the Mucosa of the Cervix and Corpus Uteri After Folliculin Administration to Intact and Spayed Rats. Zentralbl. f. Gynäk., *61*, (1937): 27–35 (Anim. Breed. Abstr., *7*, [1939]: 76).

[143] Herold, L., and Effkemann, G.: The Role of Hormonic Disturbances in the Production of Cystic Mastopathy and Epithelial Metaplasia of the Cervix. Zentralbl. f. Gynäk., *61*, (1937): 1155–1160 (Anim. Breed. Abstr., *7*, [1939]: 76–77).

[144] Herrick, J. B.: The Cytological Changes in the Cervical Mucosa of the Cow *(Bos Taurus)* Throughout the Estrous Cycle. Am. J. Vet. Res., *12*, (1951): 276–281.

[145] Hisaw, F. L., and Greep, R. O.: The Inhibition of Uterine Bleeding with Estradiol and Progesterone and Associated Endometrial Modifications. Endocrinology, *23*, (1938): 1–14.

[146] Holden, R. B.: Vascular Reactions of the Uterus of the Immature Rat. Endocrinology, *25*, (1939): 593–596.

[147] Howard, E.: Studies on the Physiology of Growth. II. The Freezing Point of the Milieux of Germ Cells and Embryonic Tissue and the Significance of Their Hypotonicity to the Adult. J. Cell. and Comp. Physiol., *3*, (1933): 291–311.

[148] Hughes, D. E.: A Study of the Diagnosis of Bovine Vibriosis with Special Reference to the Detection of Agglutinins in the Vaginal Secretions. Cornell Vet., *43*, (1953): 431–444.

[149] Iliari, G., and Calisti, V.: The Mucous Plug of Pregnancy and the Oestrous Secretion in Cows. Zootec. e Vet., *3*, (1948): 549–552 (Anim. Breed. Abstr., *17*, [1949]: 33).

[150] Ishida, K.: Morphology and Cytochemistry of Ovaries of Domestic Animals. Biol. Abstr., *30*, (1956): 738.

[151] Jeener, R.: Cytochemical Effects of Oestradiol. Nature, *159*, (1947): 578.

[152] Jeener, R.: Nucleic Acid and Phosphatase During Growth Phenomena Stimulated by Oestradiol and Prolactin. Biochem. Biophys. Acta, *2*, (1948): 439–453 (Anim. Breed. Abstr., *17*, [1949]: 188).

[153] Jepsen, A., and Vindekilde, T.: The Occurrence and Significance of Agglutinins in the Genital Organs of Brucella-Infected Cows. Am. J. Vet. Res., *12*, (1951): 97–99.

[154] Joel, C. A.: Biology of Human Sperm. Schweiz. med. Wchnschr., *72*, (1942): 440–441 (Anim. Breed. Abstr., *11*, [1943]: 263).

[155] Johnston, J. E., and Mixner, J. P.: Relationships of Hyaluronidase Concentration to Fertility of Dairy Bull Semen. J. Dai. Sci., *33*, (1950): 847–850.

[156] Knemmerer, K., and Neumann, H. G.: Reducing Substances in the Cervical Mucus of Cattle. Ztschr. tierärztl. Zucht. Biol., *58*, (1950): 416–421 (Anim. Breed. Abstr., *19*, [1951]: 59).

[157] Kamell, S. A., and Atkinson, W. B.: Effects of Ovarian Hormones on Certain Cytoplasmic Reactions in the Vaginal Epithelium of the Mouse. Proc. Soc. Exptl. Biol. and Med., *68*, (1948): 537–540 (Anim. Breed. Abstr., *17*, [1949]: 165).

[158] Kato, K.: Experimental Studies on the Agglutination of Mammalian Spermatozoa with Special Reference to Its Bearing on Fertilization. Mém. Fac. Sci. Agric. Taihokn., *19*, (1936): 1–73 (Anim. Breed. Abstr., *7*, [1939]: 374).

[159] Kaufmann, C., and Steinkamm, E.: The Effect of Prolonged Administration of Overdoses of Follicular Hormone on the Genitalia of Female Rats. Arch. f. Gynäk., *162*, (1936): 553–594 (Anim. Breed. Abstr., *5*, [1937]: 355).

[160] Kerly, M.: The Effect of the Oestrous Cycle on the Metabolism of Isolated Rat Uterus. Biochem. J., *31*, (1937): 1544–1552.

[161] Kerr, L. M. H., Campbell, J. G., and Levvy, G. A.: β-Glucuronidase as an Index of Growth in the Uterus and Other Organs. Biochem. J., *44*, (1949): 487–494.

[162] Kerr, L. M. H., Campbell, J. G., and Levvy, G. A.: Further Observations on Changes in β-Glucuronidase Activity in the Mouse. Biochem. J., *46*, (1950): 278–284.

[163] Kerr, L. M. H., and Levvy, G. A.: β-Glucuronidase as an Index of Growth in the Uterus and Other Organs. Biochem. J., *43*, (1948): 53 (Anim. Breed. Abstr., *17*, [1949]: 165).

[164] Kerr, W. R., and Robertson, M.: A Study of the Re-exposure of *Tr. Foetus* of Animals Already Exposed to the Infection as Virgin Heifers, with Some Observations on the Localization of Antibody in the Genital Tract. J. Comp. Path. and Therap., *57*, (1947): 301–313.

[165] Khayyal, M. A., and Scott, C. M.: The Oxygen Consumption of the Isolated Uterus of the Rat and Mouse. J. Physiol., *72*, (1931): 13–14.

[166] Khayyal, M. A., and Scott, C. M.: The Effect of Ovarian Extracts on the Oxygen Consumption of the Uterus. Quart. J. Exptl. Physiol., *25*, (1935): 77–83 (Anim. Breed. Abstr., *6*, [1938]: 259).

[167] Khreninger-Guggenberger, J. von: Experiments on the Vertical Migration of Sperm. Arch. f. Gynäk., *153*, (1933): 64–66 (Anim. Breed. Abstr., *1*, [1933]: 138).

[168] Kibrick, S., Belding, D. L., and Merrill, B.: Methods for the Detection of Antibodies Against Mammalian Spermatozoa. I. A Modified Macroscopic Agglutination Test. Fert. and Ster., *3*, (1952): 419–429.

[169] Kibrick, S., Belding, D. L., and Merrill, B.: Methods for the Detection of Antibodies Against Mammalian Spermatozoa. II. A Gelatin Agglutination Test. Fert. and Ster., *3*, (1952): 430–438.

[170] King, J. L.: Oxygen Usage of Uterine Muscle of the Sow. Am. J. Physiol., *99*, (1932): 631–637.

[171] Kirillov, V. S.: New Principles of the Organizations and Techniques of Mating of Cows. Probl. Zivotn., *3*, (1937): 34–42 (Anim. Breed. Abstr., *5*, [1937]: 278).

[172] Klein, M.: The Mucification of the Vaginal Epithelium in Rodents. Proc. Roy. Soc., B *124*, (1937): 23–29 (Anim. Breed. Abstr., *6*, [1938]: 165).

[173] Klein, M.: On the Simultaneous Action of Oestrone and Progesterone in Producing and Maintaining Vaginal Mucification in Rodents. Compt. rend. Soc. de biol., *127*, (1938): 918–920 (Anim. Breed. Abstr., *6*, [1938]: 259).

[174] Knobil, E.: The Relation of Steroid Hormones to β-Glucuronidase Activity. Endocrinology, *50*, (1952): 16–28.

[175] Krehbiel, R. H., and Carstens, H. P.: Roentgen Studies of the Mechanism Involved in Sperm Transportation in the Female Rabbit. Am. J. Physiol., *125*, (1939): 571–577.

[176] Kupper, G.: Electrometric Determination of the pH of Vaginal Secretions in Mares. Anim. Breed. Abstr., *10*, (1942): 145.

[177] Kurzrok, R., Leonard, S. L., and Conrad, H.: Role of Hyaluronidase in Human Fertility. Am. J. Med., *1*, (1946): 491.

[178] Kurzrok, R., Wilson, L., and Birnberg, C.: Follicu-

lar Fluid—Its Possible Role in Human Fertility and Infertility. Fert. and Ster., *4*, (1953): 479–492.

[169] Kuznecov, M. P.: The Theoretical Basis of the Methods of Introducing Sperm in Artificial Insemination of Sheep. Probl. Zivotn., *4*, (1934): 122–125 (Anim. Breed. Abstr., *3*, [1935]: 36–37).

[170] Lacassagne, A.: Progressive Modifications of Mouse Uterus Under Prolonged Influence of Estrone. Compt. rend. Soc. de biol., *120*, (1935): 1156–1158 (Anim. Breed. Abstr., *4*, [1936]: 134).

[171] Laing, J. A.: Observations on the Survival Time of the Spermatozoa in the Genital Tract of the Cow and Its Relation to Fertility. J. Agric. Sci., *35*, (1945): 72–83.

[172] Lamar, J. K., Shettles, L. B., and Delfs, E.: Cyclic Penetrability of Human Cervical Mucus to Spermatozoa *in Vitro*. Am. J. Physiol., *129*, (1940): 234–241.

[173] Lamming, G. E., and Rowson, L. E.: Ovarian Hormones and Uterine Infection in Cattle. Proc. Roy. Soc. Med., *46*, (1953): 387–392.

[174] Lardy, H. A., Pounden, W. D., and Phillips, P. H.: H-Ion Concentration of Various Fluids of the Genital Tract of the Cow. Proc. Soc. Exptl. Biol. and Med., *44*, (1940): 517–519.

[175] Larson, G. L., and Bayley, N. D.: The Fertility of Inseminations Made in Cows Showing Post-Estrous Hemorrhage. J. Dai. Sci., *38*, (1955): 549.

[176] Lawson, J. R., and MacKinnon, D. J.: *Vibrio Fetus* Infection in Cattle. Vet. Rec., *64*, (1952): 763–777.

[177] Leeb, H., and Ploberger, U.: Activity of Alkaline Phosphomonesterase in the Uterine Cervical Secretion. Biol. Abstr., *30*, (1956): 392.

[178] Lendrum, F. C., and Hisaw, F. L.: Cytology of the Monkey Endometrium Under Influence of Follicular and Corpus Luteum Hormones. Proc. Soc. Exptl. Biol. and Med., *34*, (1936): 394–396.

[179] Leonard, S. L.: The Reduction of Uterine Sperm and Uterine Fluid on Fertilization of Rat Ova. Anat. Rec., *106*, (1950): 607–615 (Anim. Breed. Abstr., *19*, [1951]: 92).

[180] Leonard, S. L., and Knobil, E.: β-Glucuronidase Activity in the Rat Uterus. Endocrinology, *47*, (1950): 331–337.

[181] Leonard, S. L., and Kurzrock, R.: A Study of Hyaluronidase Effects on the Follicle Cells of Ovulated Rat Ova. Endocrinology, *37*, (1945): 171–176.

[182] Leonard, S. L., and Periman, P. L.: Conditions Effecting the Passage of Spermatozoa Through the Utero-Tubal Junction of the Rat. Anat. Rec., *104*, (1949): 89–102.

[183] Lesbouyries, Berthelon, and Hennion: Folliculin Excretion in the Cow. Bul. Acad. vét. France, *10*, (1937): 261–265 (Anim. Breed. Abstr., *7*, [1939]: 311).

[184] Lindahl, P. E., and Kihlstrom, J. E.: On Ripening and Agglutination in Bull Spermatozoa. Second International. Cong. Physiol. Patl Anim. Rep., *1*, (1952): 70–74.

[185] Lindahl, P. E., and Kihlstrom, J. E.: An Anti-agglutinic Factor in Mammalian Sperm Plasma. Fert. and Ster., *5*, (1954): 241–255.

[186] Lindahl, P. E., and Nilsson, E.: On the Occurrence of Sperm Antagglutin in the Female Rabbit. Arch. Zool., *7*, (1954): 223–226.

[187] Lipschutz, A., and Iglesias, R.: Multiple Uterine and Extragenital Turnovers Produced by Estradiol Benzoate. Compt. rend. Soc. de biol., *129*, (1938): 519–524 (Anim. Breed. Abstr., *7*, [1939]: 78).

[188] Lombard, L., Morgan, B. B., and McNutt, S. H.: The Morphology of the Oviduct of Virgin Heifers in Relation to the Estrous Cycle. J. Morphol., *86*, (1950): 1–24.

[189] Lopyrin, A. I., and Loginova, N. V.: Rate of Movement and Time of Survival of Spermatozoa in the Genital Tract of the Ewe. Sovet. Zootech., *23*, (1939): 144–149 (Anim. Breed. Abstr., *8*, [1940]: 256).

[190] Lucas, F. V., Neufeld, H. A., Utterback, J. G., Martin, A. P., and Stotz, E.: The Effect of Estrogen on the Production of a Peroxidase in the Rat Uterus. J. Biol. Chem., *214*, (1955): 775–780.

[191] Lundgren, B.: Treatment of Purulent Endometritis in Cows with Stilbestrol. Svensk. Vet.-tidskr., *52*, (1947): 219–233.

[192] Lutwak-Mann, C.: Note on the Chemical Composition of Bovine Follicular Fluid. J. Agric. Sci., *44*, (1954): 477–480.

[193] McClean, D., and Rowlands, I. W.: Role of Hyaluronidase in Fertilization. Nature, *150*, (1942): 627–628.

[194] MacCorquodale, D. W., Thayer, S. A., and Doisy, E. A.: The Isolation of the Principal Estrogenic Substance of Liquor Folliculi. J. Biol. Chem., *115*, (1936): 435–448.

[195] MacLeod, J., and Reynolds, S. R. M.: Vascular, Metabolic and Motility Responses of Uterine Tissue Following Administration of Oestrin. Proc. Soc. Exptl. Biol. and Med., *37*, (1938): 666–668.

[196] Markee, J. E.: Rhythmic Vascular Uterine Changes. Am. J. Physiol., *100*, (1932): 32–39.

[197] Marshall, F. H. A., and Hammond, J., Jr.: Experimental Control by Hormone Action of the Oestrous Cycle in the Ferret. J. Endocrinol., *4*, (1944): 159–168.

[198] Masuda, S., Quishi, N., and Kudo, A.: Studies on Oestrus in the Cow. Res. Bull. Zootech. Exper. Sta. 56, 1950 (Anim. Breed. Abstr., *19*, [1951]: 196).

[199] Matics, S.: The Chemical Reaction of the Vaginal Mucus in Healthy, Diseased and Estrous Cattle. Anim. Breed. Abstr., *12*, (1944): 195.

[200] Mayer, D. T., Andrews, F. N., and McKenzie, F. F.: The Estrin Content of the Follicular Fluid and Urine of the Mare and Its Relation to Phenomena of the Estrual Cycle. Endocrinology, *27*, (1940): 867–872.

[201] McDonald, L. E., Black, W. G., McNutt, S. H., and Casida, L. E.: The Response of the Rabbit Uterus to Instillation of Semen at Different Phases of the Estrous Cycle. Am. J. Vet. Res., *13*, (1952): 419–424.

[202] McEntee, K., Hughes, D. E., and Gilman, H. L.: Experimentally Produced Vibriosis in Dairy Heifers. Cornell Vet., *44*, (1954): 376–394.

[203] McEuen, C. S.: Metaplasia of Uterine Epithelium Produced in Rats by Prolonged Administration of Estrin. Am. J. Cancer, *27*, (1936): 91–94 (Anim. Breed. Abstr., 7, [1939]: 379).

[204] McNutt, S. H., Schwarte, L. H., and Eveleth, D. F.: The Hydrogen Ion Concentration of Vaginal Secretions of Cows. Cornell Vet., *29*, (1939): 415–419.

[205] Merton, H.: Studies on Reproduction in the Albino Mouse. III. The Duration of Life of Spermatozoa in the Female Reproductive Tract. Proc. Roy. Soc. Edinburgh, *59*, (1939): 207–218 (Anim. Breed. Abstr., *7*, [1939]: 375).

[206] Meyer, R. K.: Relation of Glucuronidase to Action of Gonadal Hormones. Ciba Found. Col. Endocrinol., *1*, (1952): 281–282.

[207] Meyer, R. K., and Allen, W. M.: The Production of Mucified Cells in the Vaginal Epithelium of Certain Rodents by Oestrin and by Corpus Luteum Extracts. Anat. Rec., *56*, (1933): 321–344.

[208] Mirskaya, L.: The Determination of Heat in the Cow by Vaginal Smears. Probl. Zhivotn., *3*, (1933): 100–102 (Anim. Breed. Abstr., *1*, [1934]: 241).

[209] Moberg, R.: Leucocytes During the Oestrual Cycle in the Cow. Anim. Breed. Abstr., *20*, (1952): 339.

[210] Morgan, C. F.: The Effects of Androgens, Estrogens and Equine Gonadotropins on the Micin Secreting Cells on a Glycogen in the Reproductive Tract of the Female Opossum. Anat. Rec., *94*, (1946): 483.

[211] Moricard, R.: Absence of an Estrogenic Function and the Problem of a Meiogenic Function of Liquor Folliculi. Compt. rend. de l'Assoc. d. anat., *33*, (1947): 360–368 (Anim. Breed. Abstr., *18*, [1950]: 199).

[212] Moricard, R.: Penetration of the Spermatozoon *in Vitro* into the Mammalian Ovum Oxydo Potential Level. Nature, *165*, (1950): 763.

[213] Moss, S., Wrenn, T. R., and Sykes, J. F.: Alkaline Phosphatase, Glycogen, and Periodic Acid-Schiff Positive Substances in the Bovine Uterus During the Estrous Cycle. Endocrinology, *55*, (1954): 261–273.

[214] Mueller, G. C.: Incorporation of Glycine-2-C^{14} into Protein by Surviving Uteri from α-Estradiol-Treated Rats. J. Biol. Chem., *204*, (1953): 77–90.

[215] Murphey, H. S.: Studies of the Estrous or Genital Cycle of the Ox. J.A.V.M.A., *18*, (1924): 598–621.

[216] Nelson, W. O.: Endometrial and Myometrial Changes, Including Fibromyomatous Nodules, Induced in the

Uterus of the Guinea-Pig by the Prolonged Administration of Estrogenic Hormone. Anat. Rec., *68*, (1937): 99–102.

[217] Neumann, O. F., and Salzman, A. A.: The Survival and Rapidity of Penetration of Spermatozoa in the Sexual Organs of the Mare. Anim. Breed. Abstr., *13*, (1945): 10.

[218] Nicol, T., and Snell, R. S.: The Appearance of Lipoid in the Cells of the Vaginal Smear of the Guinea Pig. J. Obst. and Gynaec. Brit. Emp., *61*, (1954): 85–86 (Biol. Abstr., *29*, [1955]: 559).

[219] Nicol, T., and Snell, R. S.: The Appearance of Lipoid in the Genital Tract of the Mature Virgin Guinea Pig During Oestrous Cycle. J. Obst. Gynaec. Brit. Emp., *61*, (1954): 216–222 (Biol. Abstr., *29*, [1955]: 1345).

[220] Novazzi, G.: The Diffusion Factor (Mesomucinase, Hyaluronidase) in Domestic Animals. I. Investigations on the Organic Liquids and of Some Pregnancy Substances in Cattle. Zootec. e Vet., *5*, (1950): 1005–1014 (Anim. Breed. Abstr., *19*, [1951]: 196).

[221] Novoselov, S. I.: The Viscosity of the Mucous Secretion of the Cervix in Cattle During the Resting Stage. Anim. Breed. Abstr., *5*, (1937): 23.

[222] Odelblad, E.: Studies on the Physiology of the Follicular Fluid. Acta Endocrinol., *15*, (1954): 313–316.

[223] Ohnishi, T.: Effect of Estrogen on the Apyrase Activity of Castrated Rat Uterus. J. Fac. Sci. Tokyo Univ. Sect. 4, *7*, (1954): 51–52 (Biol. Abstr., *29*, [1955]: 1868).

[224] Oide, H.: Effect of Estrogen on the High-Energy Phosphate Content of Castrated Rat Uterus and Thymus. Annot. Zool. Japanese, *27*, (1954): 71–75 (Biol. Abstr., *29*, [1955]: 1868).

[225] Okey, R., Bloor, W. R., and Corner, G. W.: The Variations in the Lipids of the Uterine Mucosa in the Pig. J. Biol. Chem., *86*, (1930): 307–314.

[226] Olds, D.: Infertility in Cattle—A Review. J.A.V.M.A., *122*, (1953): 276–287.

[227] Olds, D., and Seath, D. M.: Factors Affecting Reproductive Efficiency in Dairy Cattle. Kentucky Agric. Exper. Sta. Bull. 605, 1954.

[228] Overholser, M. D., and Nelson, W. O.: Migration of Nuclei in Uterine Epithelium of the Monkey Following Prolonged Estrin Injections. Proc. Soc. Exptl. Biol. and Med., *34*, (1936): 839–841.

[229] Parker, G. H.: The Passage of the Spermatozoa and Ova Through the Oviducts of the Rabbit. Proc. Soc. Exptl. Biol. and Med., *27*, (1930): 826–830.

[230] Parsons, E. I., and Hyde, R. R.: An Evaluation of Spermatoxic Sera in the Prevention of Pregnancy. Am. J. Hyg., *31B*, (1939): 89–113 (Anim. Breed. Abstr., *9*, [1941]: 76).

[231] Parsutin, G. V.: Spermatozoal Viability in the Mare's Uterus. Konevodstvo, *22*, (1952): 12–19 (Anim. Breed. Abstr., *21*, [1953]: 25).

[232] Perkins, J. R., Olds, D., and Seath, D. M.: A Study of 1,000 Bovine Genitalia. J. Dai. Sci., *37*, (1954): 1158–1163.

[233] Perloff, W. H., Schultz, J., Farris, E. J., and Balin, H.: Some Aspects of the Chemical Nature of Human Ovarian Follicular Fluid. Fert. and Ster., *6*, (1955): 11–16.

[234] Phillips, R. W., and Andrews, F. N.: The Speed of Travel of Ram Spermatozoa. Anat. Rec., *68*, (1937): 127–132.

[235] Pierce, A. E.: The Demonstration of an Agglutinin to *Trichomonas Foetus* in the Vaginal Discharge of Infected Heifers. J. Comp. Path. and Therap., *57*, (1947): 84–97.

[236] Pierce, J. R., and Cope, H. B.: Crystallization of Cervical Mucus in Early Pregnancy. Relation to Abortion. Am. J. Obst. and Gynec., *5*, (1955): 815–818.

[237] Plastridge, W. N., Easterbrooks, H. L., and Williams, L. F.: The Tampon Method of Collection and the Examination of Cervicovaginal Mucus for *Vibrio Fetus* Agglutinins. J.A.V.M.A., *123*, (1953): 516–520.

[238] Poloveeva, V. V.: The Rhythmic Movements of the Uterus of the Ewe at Different Periods of the Sexual Cycle. Anim. Breed. Abstr., *10*, (1942): 105–106.

[239] Poloveeva, V. V., Okulicev, G. A., and Judovic, S. S.: Duration of Survival of Spermatozoa in the Genital Passages of the Ewe. Probl. Zivotn., *1*, (1938): 169–172 (Anim. Breed. Abstr., *8*, [1940]: 52).

[240] Pommerenke, W. T., and Viergiver, E.: Cervical Mucus and the Menstrual Cycle, in "The Problem of Fertility." E. T. Engle (ed.), Princeton University Press, Princeton, N.J., 1946.

[241] Quinlan, J., Mare, G. S., and Roux, L. L.: A Study of the Duration of Motility of Spermatozoa in the Different Divisions of the Reproductive Tract of the Merino Ewe. Onderstepoort J. Vet Sci. and Anim. Indust., *1*, (1933): 136–145 (Anim. Breed. Abstr., *1*, [1934]: 250–251).

[242] Quinlan, J., Myburgh, S. J., and De Vos, D.: The Hydrogen-Ion Concentration of the Vaginal Secretion of Merino Sheep During Estrus, Diestrus and Pregnancy, with Some Remarks on Its Influence on Sex Determination, and the Influence of Vaginal Temperature at the Time of Mating on Conception. Onderstepoort. J. Vet. Sci. and Anim. Indust., *17*, (1941): 105–114 (Anim. Breed. Abstr., *11*, [1943]: 236).

[243] Raps, G.: Effect of Mucous Volume on Conception. J.A.V.M.A., *109*, (1946): 275–276.

[244] Ras Crotto, N.: The Vaginal Epithelium of the Cow. Its Cytological Modifications. Rev. méd. vét., *34*, (1952): 91–98 (Anim. Breed. Abstr., *21*, [1953]: 256).

[245] Reese, J. D.: Cyclic Variation in Certain Oxmiophile Granules in the Rat Uterus. Anat. Rec., *48*, (1931): 55–64.

[246] Reynolds, S. R. M.: Acetylcholine Content of Uteri Before and After Administration of Oestrin to Ovariectomized Rabbits. J. Physiol., *95*, (1939): 258–268.

[247] Ring, J. R.: Changes in Alkaline Phosphatase Activity of Rat Vaginal Epithelium During the Estrous Cycle. Anat. Rec., *107*, (1950): 121–132.

[248] Ristic, M., Sanders, D. A., and Tyler, M. E.: *Vibrio Fetus* Infection in Cattle. I. A Comparative Study of the Agglutinin Response in Cervicovaginal Mucus and Blood Serum to Homologous Antigens Following Abortion. Am. J. Vet. Res., *16*, (1955): 246–250.

[249] Roark, D. B., and Herman, H. A.: Physiological and Histological Phenomena of the Bovine Estrual Cycle with Special Reference to Vaginal-Cervical Secretions. Missouri Agric. Exper. Sta. Res. Bull. 455, 1950.

[250] Roberts, S., and Szego, C. M.: Steroid Interaction in the Metabolism of Reproductive Target Organs. Physiol. Rev., *33*, (1953): 593–629.

[251] Ross, V., Miller, E. G., Jr., and Kurzrok, R.: Metabolism of Human Sperm. Endocrinology, *28*, (1941): 885–893.

[252] Rossman, I.: Uterine Contractions and the Transport of Sperm in the Rat. Anat. Rec., *69*, (1937): 133–149.

[253] Rowlands, I. W.: Capacity of Hyaluronidase to Increase the Fertilizing Power of Sperm. Nature, *154*, (1944): 332–333.

[254] Rowson, L. E. A., Lamming, G. E., and Fry, R. M.: The Relationship Between Ovarian Hormones and Uterine Infection. Vet. Rec., *65*, (1953): 335–340.

[255] Rowson, L. E. A., Lamming, G. E., and Fry, R. M.: Influence of Ovarian Hormones on Uterine Infection. Nature, *171*, (1953): 749.

[256] Rubenstein, B. B., Strauss, H., Lazarus, M. L., and Haukin, H.: Sperm Survival in Women. Motile Sperm in the Fundus and Tubes of Surgical Cases. Fert. and Ster., *2*, (1951): 15–19.

[257] Rumjancev, N. V., and Flegmatov, N. A.: On the Influence of Secretions of Female Genital Organs on Spermatozoa. Trudy Mosk. Zoovet. Inst., *2*, (1935): 163–175 (Anim. Breed. Abstr., *6*, [1938]: 254–255).

[258] Sabin, M., and Latour, J. P. A.: A Test for the Rapid Diagnosis of Secretory Endometrium. Fert. and Ster., *5*, (1954): 44–51.

[259] Sallman, B., and Birkeland, J. M.: Interrelationships of Spermatozoa Count, Hyaluronidase Titer and Fertilization. Am. J. Physiol., *152*, (1948): 271–279.

[260] Salvatore, C. A.: Action of Estrone and Progesterone on Nuclear Volume (Studied by Applying the Karyometric-Statistical Method). Biol. Bull., *99*, (1950): 112–119 (Anim. Breed. Abstr., *19*, [1951]: 95).

[261] Sawyer, C. H., and Everett, J. M.: Effects of Various Hormonal Conditions in the Intact Rat on the Syn-

thesis of Serum Cholinesterase. Endocrinology, *39*, (1946): 307–322.

[203] Schatalow, P.: The Sexual Cycle of the Horse and Cow Studied by the Vaginal Smear. Berl. tierärztl. Wchnschr., *49*, (1933): 81–84 (Anim. Breed. Abstr., *1*, [1933]: 155).

[204] Schilling, E., and Kordts, E.: The Migration of Spermatozoa in the Rabbit Uterus. Ztschr tierärztl. Zucht. Biol., *60*, (1952): 73–78 (Anim. Breed. Abstr., *20*, [1952]: 275).

[205] Schneerson, S. S.: Vaginal Smears as a Method of Determining Heat in Cows. Anim. Breed. Abstr., *4*, (1936): 419.

[206] Schockaert, J. A., and Delrue, G.: Action of Folliculin on the Vaginal pH. Compt. rend. Soc. de biol., *123*, (1936): 306–308 (Anim. Breed. Abstr., *4*, [1936]: 503).

[207] Schoop, G., and Klette, H.: Gehaufte Sterilitat durch oestrogene Staffe im Weidegras. Second Internat. Cong. Physiol. and Path. Anim. Reprod. and Artif. Insemin., Copenhagen, 1952.

[208] Schott, R. G., and Phillips, R. W.: Rate of Sperm Travel and Time of Ovulation in Sheep. Anat. Rec., *79*, (1941): 531–540.

[209] Scott Blair, G. W., Folley, S. J., Coppen, F. M. V., and Malpress, F. H.: Rheological Properties of Bovine Cervical Secretions Durnig the Oestrus Cycle. Nature, *147*, (1941): 453–454.

[210] Scott Blair, G. W., Folley, S. J., Malpress, F. H., and Coppen, F. M. V.: Variations in Certain Properties of Bovine Cervical Mucus During the Oestrous Cycle. Biochem. J., *35*, (1941): 1039–1049.

[211] Scott Blair, G. W., and Glover, F. A.: Farm Testing for Bovine Pregnancy, Oestrus and Subfertility. Agriculture, *62*, (1955): 16–19.

[212] Selye, H., Thomson, D. L., and Collip, J. B.: Metaplasia of Uterine Epithelium Produced by Chronic Estrin Administration. Nature, *135*, (1935): 65–66.

[213] Sergin, N. P., Kuzuecov, M. P., Kozlova, V. M., and Nesmejanova, T. N.: Physico-Chemical Conditions in the Genital Tract of the Cow and Survival of Spermatozoa. Dokl. Akad. Seljskohoz. Nauk., *15*, (1940): 24–28 (Anim. Breed. Abstr., *9*, [1941]: 18–19).

[214] Skaarup Thygesen, A.: pH Determination in Uterine Mucus from Freshly Slaughtered Cows with Special Regard to the Demonstration of Endometritis. Maanedskr. Dyrl., *59*, (1947): 281–290.

[215] Skaarup Thygesen, A.: Determinations of pH in the Uterine Mucus, etc., and Occurrence of Adhesions in the Region of the Ovaries in Cattle. Med. Danske Dyrl., *31*, (1948): 407–414.

[216] Smirnov-Ugrjumov, D., and Sain, S.: The Method of Ascertaining Heat by Means of Vaginal Smears. Socialist. Zivotn., *1*, (1935): 41–43 (Anim. Breed. Abstr., *4*, [1936]: 185).

[217] Smith, A. U.: Some Antigenic Properties of Mammalian Spermatozoa. Proc. Roy. Soc., B *136*, (1949): 46–66 (Anim. Breed. Abstr., *17*, [1949]: 279).

[218] Smith, A. U.: The Antigenic Relationship of Some Mammalian Spermatozoa. Proc. Roy. Soc., B *136*, (1949): 472–479 (Anim. Breed. Abstr., *17*, [1949]: 393).

[219] Smith, A. U.: Fertilization *in Vitro* of the Mammalian Egg. Biochem. Soc. Symp., *7*, (1951): 3–10.

[220] Smith, S. E., and Asdell, S. A.: The pH of Vaginal Mucus of Normal and Sterile Dairy Cows. Am. J. Vet. Res., *2*, (1941): 167–174.

[221] Soderwall, A. L., and Blandau, R. J.: The Duration of the Fertilizing Capacity of Spermatozoa in the Female Genital Tract of the Rat. J. Exptl. Zool., *88*, (1941): 55–64.

[222] Soderwall, A. L., and Young, W. C.: The Effect of Aging in the Female Genital Tract on the Fertilizing Capacity of Guinea Pig Spermatozoa. Anat. Rec., *78*, (1940): 19–29.

[223] Sokolovskaja, I. I.: The Effect of Semen on the Mucin of the Animal Organism. Dokl. Akad. Seljskohoz. Nauk. Lenin, *1950*, (1950): 12–15 (Anim. Breed. Abstr., *18*, [1950]: 172).

[224] Sokolovskaja, I. I., Rodin, I. I., Smirnov-Ugrjumov, D. V., and Tjupic, M. M.: The Use of Mucinase for Increasing Fertilization of Cows. Sovet. Zootech., *1950*, (1950): 65–74 (Anim. Breed. Abstr., *18*, [1950]: 404).

[225] Spyker, M. A., and Fidler, R. S.: Glycogen Studies on Human Endometrium. J. Clin. Endocrinol., *2*, (1942): 365–368.

[226] Starke, N. C.: The Sperm Picture of Rams of Different Breeds as an Indication of Their Fertility. II. The Rate of Sperm Travel in the Genital Tract of the Ewe. Onderstepoort J. Vet. Sci. and Anim. Indust., *22*, (1949): 514–525 (Anim. Breed. Abstr., *18*, [1950]: 296).

[227] Stein, I. F., and Cohen, M. R.: Sperm Survival at Estimated Ovulation Time. Prognostic Significance. Fert. and Ster., *1*, (1950): 169–175.

[228] Sturgis, S. H.: Method for Obtaining Uterine Fluid from the Monkey: Effect of Pilocarpine, Atropine, Physiological Salt Solution and Adrenalin. Endocrinology, *31*, (1942): 664–672.

[229] Sumner, J. B., and Myrback, K.: The Enzymes. Vol. I, Part 1. Academic Press, New York, 1950.

[230] Supperer, R.: Investigations on the Occurrence of Estrogenic Substances in the Vaginal Secretion of Cattle During Estrus. Dissertation, Berlin, 1942 (Anim. Breed. Abstr., *13*, [1945]: 86–87).

[231] Sweeney, B. M.: The Effect of Estrone on Anaerobic Glycolysis of the Uterus of the Rat *in Vitro*. J. Lab. and Clin. Med., *29*, (1944): 957–962.

[232] Swyer, G. I. M.: A Tubal Factor Concerned in the Denudation of Rabbit Ova. Nature, *159*, (1947): 873–874.

[233] Szego, C. M., and Roberts, S.: Steroid Action and Interaction in Uterine Metabolism. Rec. Prog. Hormone Res., *8*, (1953): 419–469.

[234] Talbot, N. B., Lowry, O. H., and Astwood, E. B.: Influence of Estrogen on the Electrolyte Pattern of the Immature Rat Uterus. J. Biol. Chem., *132*, (1940): 1–9.

[235] Tanabe, T. Y., and Almquist, J. O.: Some Cases of Infertility in Dairy Heifers. J. Dai. Sci., *36*, (1953): 586.

[236] Tanabe, T. Y., and Casida, L. E.: The Nature of Reproductive Failures in Cows of Low Fertility. J. Dai. Sci., *32*, (1949): 237–246.

[237] Telfer, M. A.: Influence of Estradiol on Nucleic Acids, Respiratory Enzymes, and the Distribution of Nitrogen in the Rat Uterus. Arch. Biochem. and Biophys., *44*, (1953): 111–119.

[238] Thibault, C., and Soulairac, A.: Phosphatase Activity of the Genital Tract of Mammals. Experimental Modifications in the Female Rat. Ann. Endocrinol., *9*, (1948): 555–557 (Anim. Breed. Abstr., *18*, [1950]: 89).

[239] Tribby, C. L.: The Intracellular Lipin, Mucoid, and Glycogen of the Vaginal Epithelium of the Guinea Pig. Anat. Rec., *86*, (1943): 425–451.

[240] Trimberger, G. W.: Menstruation Frequency and Its Relation to Conception in Dairy Cattle. J. Dai. Sci., *24*, (1941): 819–823.

[241] Trimberger, G. W., and Davis, H. P.: Conception Rate in Dairy Cattle by Artificial Insemination at Various Stages of Estrus. Nebraska Agric. Exper. Sta. Res. Bull. 129, 1943.

[242] Urdan, B. E., and Kurzon, A. M.: Cyclic Changes in Cervical Mucus. Am. J. Obst. and Gynec., *5*, (1955): 3–12.

[243] VanDemark, N. L., Engberson, R. D., and Moeller, A. N.: Unpublished data, 1950.

[244] VanDemark, N. L., and Hays, R. L.: Rapid Sperm Transport in the Cow. Fert. and Ster., *5*, (1954): 131–137.

[245] VanDemark, N. L., and Hays, R. L.: Sperm Transport in the Perfused Genital Tract of the Cow. Am. J. Physiol., *183*, (1955): 510–512.

[246] VanDemark, N. L., and Moeller, A. N.: Spermatozoan Transport in the Reproductive Tract of the Cow. J. Dai. Sci., *33*, (1950): 390–391.

[247] VanDemark, N. L., and Moeller, A. N.: Speed of Spermatozoan Transport in Reproductive Tract of Estrous Cow. Am. J. Physiol., *165*, (1951): 674–679.

[248] Vandeplassche, M., and Paredis, F.: Preservation of the Fertilizing Capacity of Bull Semen in the Genital Tract of the Cow. Nature, *162*, (1948): 813.

[209] Vandeplassche, M., and Paredis, F.: How Long Does Bull Sperm Maintain Its Fertilizing Capacity in the Genital Tract of the Cow? Tijdschr. Diergeneesk., *74*, (1949): 831–838 (Anim. Breed. Abstr., *19*, [1951]: 63).

[210] VanDyke, H. B., and Chen, G.: Observations on the Biochemistry of the Genital Tract of the Female Macaque Particularly During the Menstrual Cycle. Am. J. Anat., *58*, (1936): 473–500.

[211] VanKlinkenberg, G. A.: Extremely High Alkaline Phosphatase Activity in the Vaginal Mucus of the Cow. Nature, *172*, (1953): 397.

[212] Verna, J. E., Wasserman, B., and Henderson, B. W.: The Agglutination Test in the Study of the Microflora of the Bovine Vulvo-Vaginal Mucosa. (abstr.) J. Dai. Sci., *37*, (1954): 671.

[213] Vokaer, R.: Is the Glycogen Content of Female Vaginal Cells a Test for Estrogenic Activity? Ann. Endocrinol., *13*, (1952): 500–504 (Biol. Abstr., *28*, [1954]: 823).

[214] Wade, R., and Jones, H. W., Jr.: Inhibition of Human Endometrial Adenosine Triphosphatase by Progesterone. Am. J. Obst. and Gynec., *3*, (1954): 608–614.

[215] Warren, M. R.: Observations on the Uterine Fluid of the Rat. Am. J. Physiol., *122*, (1938): 602–608.

[216] Washko, F. V., Donham, C. R., and Heimlich, A.: Response of Cattle to Brucella Following Sexual Stimulation and Brucella Exposure During Early Calfhood. Am. J. Vet. Res., *13*, (1952): 437–440.

[217] Watson, M. C.: The Effect of Cervical Secretions on the Vitality of Spermatozoa. Canad. M. A. J., *40*, (1939): 542–543 (Anim. Breed. Abstr., *8*, [1940]: 311).

[218] Weber, A. F., Morgan, B. B., and McNutt, S. H.: A Histological Study of Metrorrhagia in the Virgin Heifer. Am. J. Anat., *83*, (1948): 309–327.

[219] Weber, A. F., Morgan, B. B., and McNutt, S. H.: Clinical and Postmortem Observations on Metrorrhagia in the Virgin Heifer. North Am. Vet., *29*, (1948): 705–710.

[220] Weber, A. F., Morgan, B. B., and McNutt, S. H.: Perimetrial Hemorrhage in Virgin Heifers. Cornell Vet., *39*, (1949): 261–265.

[220] Weber, A. F., Morgan, B. B., and McNutt, S. H.: Tissue Mast Cells in the Virgin Bovine Uterus During the Estrous Cycle. Cornell Vet., *40*, (1950): 34–38.

[221] Weeth, H. J.: A Histological and Histochemical Study of the Bovine Oviducts, Uterus and Placenta. Dissertation, Univ. of Missouri, 1952 (Biol. Abstr., *27*, [1953]: 6F).

[222] Weeth, H. J., and Herman, H. A.: A Histological and Histochemical Study of the Bovine Oviducts, Uterus and Placenta. Missouri Agric. Exper. Sta. Bull. 501, 1952.

[223] Werner, I.: Studies on Glycoproteins from Mucous Epithelium and Epithelial Secretions. Acta Soc. Med. Upsaliensis, *58*, (1953): 1–55 (Biol. Abstr., *29*, [1955]: 1563).

[224] Wilkins, W.: Sodium Potassium, Calcium, Magnesium and Phosphorus Content of Skeletal and Cardiac Muscle, Bladder and Uterus. Proc. Soc. Exptl. Biol. and Med., *31*, (1934): 1117–1118.

[225] Williams, M. F.: The Vascular Architecture of the Rat Uterus as Influenced by Estrogen and Progesterone. Am. J. Anat., *83*, (1948): 247–307.

[226] Woodman, H. E., and Hammond, J.: The Mucous Secretion of the Cervix of the Cow. J. Agric. Sci., *15*, (1925): 107–124.

[227] Wrenn, T. R., Moss, S., and Sykes, J. F.: The Effect of Estrogen and Progesterone on the Distribution of Alkaline Phosphatase and Glycogen in the Bovine Uterus. (abstr.) J. Dai. Sci., *37*, (1954): 671.

[228] Wunsche, K.: The Behavior of Spermatozoa in the Oestrual Mucus of the Cow. Wien. tierärztl. Monatschr., *39*, (1952): 545–548 (Anim. Breed. Abstr., *21*, [1953]: 257).

[229] Yamada, F.: Studies on the Ciliary Movements of the Oviducts. Jap. J. Physiol., *2*, (1952): 194–197.

[230] Zittle, C. A., and O'Dell, R. A.: Chemical Studies of Bull Spermatozoa, Lipid, Sulphur, Cystine, Nitrogen, Phosphorus, and Nucleic Acid Content of Whole Spermatozoa and of Parts Obtained by Physical Means. J. Biol. Chem., *140*, (1941): 899–907.

[231] Zondek, B.: The Effect of Prolonged Application of Large Doses of Follicular Hormone on the Uterus of Rabbits. J. Exptl. Med., *63*, (1936): 789–794 (Anim. Breed. Abstr., *4*, [1936]: 505).

[232] Zondek, B.: The Effect of Long-Continued Large Doses of Follicle Hormone upon the Uterus of the Rat. Am. J. Obst. and Gynec., *33*, (1937): 979–988 (Anim. Breed. Abstr., *6*, [1938]: 355–356).

[233] Zondek, B.: Cervical Mucus in Pregnancy. Inability of Estrogen to Produce Aborization in Pregnancy and Its Clinical Significance. Am. J. Obst. and Gynec., *4*, (1954): 484–491.

[234] Zondek, B., and Rozin, S.: Cervical Mucus Aborization. Its Use in the Determination of Corpus Luteum Function. Am. J. Obst. and Gynec., *3*, (1954): 463–470 (Biol. Abstr., *29*, [1955]: 312).

[235] Zuckerman, S., Palmer, A., and Bourne, G.: Changes in the Water-Content of Organs and Tissues as a Result of Stimulation by Oestradiol. Nature, *143*, (1939): 521–522.

[236] Zuckerman, S., Palmer, A., and Hanson, D. A.: The Effect of Steroid Hormones on the Water Content of Tissues. J. Endocrinol., *6*, (1950): 261–276.

The Behavior of Spermatozoa in Luminal Fluids of Bovine Female Genitalia

DURWARD OLDS, D.V.M., Ph.D., and N. L. VanDEMARK, Ph.D.

Urbana, Illinois

LUMINAL FLUIDS of female genital organs may, during various phases of the reproductive process, serve as vehicles for the transport of spermatozoa, mediums for the preservation of spermatozoan viability, and they no doubt have other functions associated with fertilization and embryonic development.

It has been reported that spermatozoa may remain fertile in the female tract of cattle for 15 to 56 hours.[2, 14, 16, 34, 35] Beshlebnov [2] found that survival of sperm was longest in the cervix (24 to 30 hours), whereas sperm in the vagina were immobilized after four hours. In the uterus and oviducts, degeneration occurred after ten to 15 hours. No explanation for the variation in survival is known. It is possible that knowledge of the composition of the fluids in the various parts of the tract would be suggestive of their ability to support sperm.

The exact composition of the cervicovaginal mucin is not known, but it is presumably a polysaccharide or glycoprotein similar to hyaluronic acid compounds.[11] There are conflicting reports on whether hyaluronidase acts on cervicovaginal mucus. Kaemmerer and Neumann [13] reported that the addition of hyaluronidase increased the free reducing substances in mucus. However, Boyland,[3] McClean and Rowlands,[22] and Calisti [5] found no effect of hyaluronidase on mucus. Lipphardt and Pommerenke [18] reported that the incubation of washed spermatozoa in human cervical mucus resulted in a decrease in the total reducing substances which were present after acid hydrolysis. The addition of "mucinase" (hyaluronidase) to diluted bull semen has been reported to increase fertility by 18 to 20 percentage units.[1, 31, 32] These authors believed that the effect probably was due to a reduction in the viscosity of the mucus. Calisti and Quacquarini [6] reported that the addition of hyaluronidase to high dilutions of bull semen had no beneficial action on fertility. Chang [7] found that the application of hyaluronidase to the vagina before insemination or the addition of hyaluronidase to the sperm suspension had no significant effect on the fertilization of rabbit ova *in vivo*. Fiorentino and Caretta [10] obtained inconclusive results from the addition of hyaluronidase to bull semen; 3 bulls showed slight increases in fertility, while 1 bull showed a decrease. Johnston and Mixner [12] found no significant correlation between the fertility of bull semen and its hyaluronidase content. Sallman and Birkeland [29] presented evidence that high concentrations of hyaluronidase in semen were, in fact, associated with decreased fertility.

Bull spermatozoa have been reported to live in follicular fluid for as long as 48 hours.[28] However, follicular fluid from nonestrous cows caused agglutination and death of the spermatozoa. Kurzrok *et al.*[15] found that human follicular fluid would maintain human spermatozoa for a longer time and in greater activity than seminal plasma. Similarly, Perloff *et al.*[26] found that all samples of human follicular fluid increased sperm motility. The addition of liquor folliculi to uterine mucus and semen *in vitro* prolonged spermatozoan viability.[25] However, Brown [4] and Ross *et al.*[27] reported that sperm motility was not noticeably affected by follicular fluid. Lutwak-Mann [19] examined the effect of follicular fluid on sperm fructolysis and concluded that there was no effect other than that due to the glucose content.

The present study was made in an effort to determine the duration of motility and metabolic activity of spermatozoa in cervicovaginal mucus, uterine fluid, oviduct fluid, and follicular fluid. Data on the chemical composition of these fluids is being published elsewhere.[24]

MATERIALS AND METHODS

Reproductive organs were obtained from 16 cows slaughtered at known stages of the estrous

From the Department of Dairy Science, University of Illinois, Urbana.

This article is part of a dissertation submitted, while the senior author was an AVMA fellow at the University of Illinois, in partial fulfillment of the requirements for the Ph.D. degree.

Dr. Olds' present address is Dairy Section, University of Kentucky, Lexington.

cycle and from 18 cows at unknown stages. Cervicovaginal mucus, uterine fluid, oviduct fluid, and follicular fluid were collected from these organs and used as diluents for bull semen. A hand-operated clothes wringer was used in the collection of uterine and oviduct fluids. It was not always possible to collect samples of all four fluids from the organs of each individual cow and, in the case of the cows slaughtered at unknown stages of the cycle, the respective samples were generally pooled. Semen was used from 8 different bulls.

Capillary pipettes about 1.2 mm. in diameter were drawn from 7.0-mm. glass tubing. A small amount of the fluid under study was drawn into the pipette; this was followed by a small air bubble and a small amount of semen. The proportion of semen to diluent was approximately 1:3 or 1:4.

The large end of the pipette was removed, leaving a capillary tube about 3 inches long which would fit in the mechanical stage of a microscope. Both ends of the capillary tube were plugged with vaseline. The rate at which spermatozoa traveled in the test fluid was estimated by determining the distance traveled through the capillary tube in a given length of time. The loaded capillary tubes were then placed in a 37 C. incubator and examined periodically for motility of the spermatozoa.

The effect of testicular hyaluronidase on the reducing power, viscosity, and ability of cervicovaginal mucus to maintain viability of spermatozoa was studied. The hyaluronidase was dissolved in a 0.02 *M* phosphate buffer of pH 6.8 containing 0.45 per cent NaCl and 0.01 per cent bovine albumin.[8] The concentration of enzyme was about 150 turbidity-reducing units per milliliter. For viability determinations, the solution was made isotonic by the addition of NaCl. Total reducing sugars were determined for mixtures of 1.0 ml. of mucus and 1.0 ml. of buffer or buffer-containing hyaluronidase after incubation for two hours at 37 C. In some trials, *beta* glucuronidase was also added to the hyaluronidase solution. In other trials, the solution of purified enzymes was replaced by seminal plasma which had been dialyzed against distilled water and then against physiological saline. Viscosity determinations were made on various dilutions of mucus with the above buffer solution in an Ostwald viscosimeter. Dispersion was accomplished in a Waring blendor.

Oxygen uptake by spermatozoa in cervicovaginal mucus, uterine fluid, oviduct fluid, and follicular fluid was determined in a Warburg respiration apparatus. Mucus and oviduct fluid were diluted 1:9 with physiological saline before use; uterine fluid was diluted 1:3 with saline. Semen and the above fluids were mixed in a proportion of 1:4 when placed in the Warburg flasks. Reducing sugars, lactic acid, and pH were determined before and after three and one-half hours of incubation in the Warburg apparatus. The reducing sugars were determined with Somogyi's copper-carbonate-tartrate[9] and Nelson's arseno-molybdate.[10] Lactic acid was determined by the method of Barker and Summerson.[11]

RESULTS

On the average, under relatively anaerobic conditions at 37 C., spermatozoa remained motile about nine hours in cervicovaginal mucus, seven hours in uterine fluid, 12 hours in oviduct fluid, and 19 hours in follicular fluid. Though the design of the experiment was not suitable for segregating variance due to differences in individual cows and in ejaculates, an analysis of variance showed the differences between fluids and the differences between stages of the cycle within fluids to be highly significant. In general, spermatozoa lived longer in mucus and uterine fluid from cows in or near estrus than in the same fluids at other stages of the cycle. However, limited data suggest that viability in oviduct fluid was enhanced by fluids obtained at 11 to 17 days postestrus. Stage of cycle appeared to have no effect on the ability of follicular fluid to maintain viable spermatozoa. These data are summarized (table 1).

TABLE 1—Viability of Spermatozoa in Genital Tract Fluids Under Anaerobic Conditions at 37 C.

Stage of cycle*	Fluid samples from: Vagina No.	Vagina Hr.	Uterus No.	Uterus Hr.	Oviduct No.	Oviduct Hr.	Follicle No.	Follicle Hr.
1	10	10.9	5	9.6	2	8.0	7	19.9
2	1	2.0	0	—	1	6.0	3	18.7
3	6	5.8	4	5.5	5	15.0	7	17.9
Unknown	1	10.0	3	5.3	0	—	1	12.0
Average	18	8.7	12	7.2	8	12.1	18	18.9

* Stage 1 refers to cows slaughtered between 18 days postestrus and three days postestrus (*i.e.*, in or near heat); stage 2 refers to cows between four and ten days postestrus; stage 3, between 11 and 17 days postestrus.

The rate at which spermatozoa penetrated the genital tract fluids was determined at room temperature. In general, the spermatozoa progressed at the rate of about 3 to 4 mm. per minute in mucus and oviduct fluid and about 6 mm. per minute in uterine and follicular fluids. There appeared to be little difference in the rate of travel in mucus or follicular fluid due to stage of cycle. However, the rate appeared to be markedly faster in uterine and oviduct fluids from cows near estrus. Though these differences were statistically highly significant, difficulties in measurement may have introduced considerable

error, especially in the case of cervicovaginal mucus.

Oxygen uptake, reducing sugar utilization, and lactic acid accumulation were determined for two semen samples incubated three and one-half hours at 37.5 C. in each of the four genital tract fluids which were collected and pooled from 8 cows slaughtered at various unknown stages of the cycle. Control samples containing saline instead of semen were included so that corrections could be made when necessary for metabolic activity in the natural fluids in the absence of spermatozoa. These corrections were generally small. Semen was added to a saline diluent as a further control. This arrangement of controls made it possible to check on reliability of the chemical analyses, since quantities of reducing sugar or of lactic acid found in the mixtures of semen and natural fluid should be equal to the sum of the amount found in the semen alone and in the natural fluid alone. There were some discrepancies in these values, but the errors were both positive and negative in direction.

It was found that oxygen uptake by the spermatozoa was highest in follicular fluid followed by oviduct fluid, mucus, and uterine fluid, respectively (fig. 1). It may be noteworthy that this is the same decreasing order shown by these fluids in maintaining the viability of spermatozoa.

It is apparent from data for these semen samples that the metabolic behavior of the sperm varied greatly in the different fluids (table 2). Glucose utilization was enhanced in the presence of follicular fluid and was reduced in uterine and oviduct fluids. On the other hand, oxygen consumption was reduced by mucus and uterine fluid and was slightly increased by oviduct and follicular fluid. The reasons for the differences in metabolic response are not understood at present and should be considered in future research.

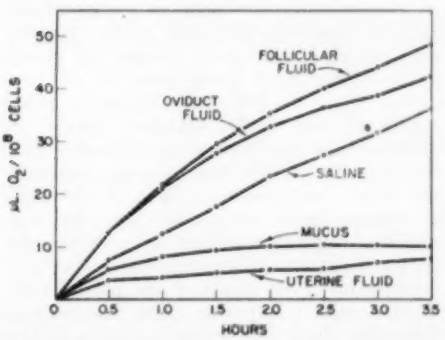


Fig. 1—Average oxygen uptake by two semen samples in genital tract fluids pooled from 8 cows.

TABLE 2—Average Glucose Utilization, Lactic Acid Production, and Oxygen Uptake by Spermatozoa of Two Semen Samples in Genital Tract Fluids (3.5 Hours at 37.5 C.)

Diluent	Metabolites (micromoles)		
	Glucose utilized	Lactic acid accumulated	Oxygen uptake
Mucus	1.57	3.77	1.19
Uterine fluid	0.52	−0.02	0.49
Oviduct fluid	0.54	2.74	4.75
Follicular fluid	4.2	4.26	5.75
Saline	1.74	1.58	4.28

The pH of the fluids which contained semen, at the end of the 3.5-hour experimental period in the Warburg flask, was: follicular fluid, 7.1; uterine fluid, 6.2; oviduct fluid, 6.1; and mucus, 5.3. In contrast, the fluids containing saline instead of semen had pH values as follows after incubation: mucus, 9.6; follicular fluid, 9.3; oviduct fluid, 6.8; and uterine fluid, 6.6. Spermatozoa were nearly all dead in mucus and uterine fluid, but many were living in oviduct and follicular fluid. In general, motility was better in saline than in any of the natural fluids. The data indicate that lactic acid was not oxidized appreciably in mucus. It may be that oxygen does not diffuse readily into mucous solutions. There may also be differences in the buffering capacity of the genital tract fluids. In live cows, there may be compensating factors which would prevent any marked accumulation of metabolic products and pH changes.

In six of 13 experiments, it appeared that hyaluronidase increased the reducing sugars of mucus from about 6.85 mg. to about 14.76 mg. per 100 ml. In the other experiments, there was no appreciable change. The presence of *beta* glucuronidase, in addition to hyaluronidase, did not affect the results. The treatment of mucus with dialyzed seminal plasma did not affect the level of reducing sugars during a period of two hours' incubation at 37 C. Mucus diluted 1:1 with a 0.02 *M* phosphate buffer of pH 6.8 and made isotonic with NaCl appeared to maintain viability of spermatozoa about one and one-half hours longer than untreated mu-

cus. The addition of hyaluronidase to the buffer and mucous mixture resulted in a further prolongation of spermatozoan viability (average, about 1 hour). These differences were small but statistically significant. The viscosity of mucus did not appear to be affected by treatment with hyaluronidase.

Five additional experiments were conducted in an effort to determine how follicular fluid caused increased oxygen uptake by spermatozoa. In two experiments, oxygen uptake of spermatozoa was compared in saline, yolk citrate, and in follicular fluid. In each case, oxygen uptake was greatest in follicular fluid followed by saline and yolk citrate, respectively. In view of the relatively low K concentration of follicular fluid, two experiments were conducted in which follicular fluid was diluted 1:1 with either 0.9 per cent NaCl or 1.14 per cent KCl before mixing with semen (1:4) in the Warburg flasks. There was no evidence that increasing the K concentration prevented the stimulation of oxygen uptake by spermatozoa in follicular fluid. In another experiment, follicular fluid was dialyzed against distilled water and then against either saline or saline plus glucose (50 mg./100 ml.). Follicular fluid retained its stimulating action regardless of whether it was undialyzed or dialyzed against either saline or saline plus glucose. This indicates that the effect on sperm metabolism is due to nondialyzable substances such as enzymes, but further experiments should be conducted before this conclusion is drawn with certainty.

Agglutination of spermatozoa was frequently noted in all of the genital tract fluids studied, but most frequently in follicular fluid. No cyclic variation in the occurrence of agglutination was apparent, though it is possible that agglutination does not occur or occurs less frequently at certain stages of estrus. In follicular fluid, agglutination of spermatozoa was observed to occur within ten minutes at room temperature while, in other cases, it occurred only after three to four hours at 37 C. The spermatozoa aggregated in clusters of varying numbers and the tails remained motile. There were always some free spermatozoa not involved in the agglutination. It was frequently noted that agglutination was absent in the capillary tubes near the junction with semen, though it was extensive further away from the semen. This may have been due either to the gradual arrival of "fresh" spermatozoa or to the diffusion of an antiagglutinating factor which has been reported in seminal plasma.[17] In the case of uterine fluid and especially oviduct fluid, there was frequently an adhesion between spermatozoa and cellular fragments in the female tract fluids. The significance of these agglutination phenomena is not known. Spermatozoa have been found to contain antigens similar or identical to those of red blood cells.[9] Whether cows gradually develop antibodies toward those antigens of spermatozoa, which are foreign to their own cellular antigens, during repeated inseminations is also unknown. As a speculation, this agglutination might be a means of preventing the occurrence of a pregnancy in which the fetus would possess genetic antigens foreign to those of the dam. Evidence of the occurrence of "intrauterine selection" in women has been reported.[20, 21, 30] Another speculation is that this agglutination process might be involved in the normally occurring union of ova and spermatozoa.

SUMMARY

Reproductive organs were obtained from 16 cows slaughtered at known stages of the estrous cycle and from 18 cows at unknown stages. Cervicovaginal mucus, uterine fluid, oviduct fluid, and follicular fluid were collected from these organs and used as diluents for bull semen. On the average, under relatively anaerobic conditions at 37 C., spermatozoa remained motile about nine hours in mucus, seven hours in uterine fluid, 12 hours in oviduct fluid, and 19 hours in follicular fluid. In general, spermatozoa lived longer in mucus and uterine fluid from cows in or near estrus than in the same fluids at other stages of the cycle. Oxygen uptake by spermatozoa was highest in follicular fluid followed by oviduct fluid, mucus, and uterine fluid, respectively. This is the same decreasing order shown by these fluids in maintaining the viability of spermatozoa. Agglutination of spermatozoa was frequently observed in all of the genital tract fluids studied but was most frequent in follicular fluid. No cyclic variation in this respect was apparent.

References

[1] Aslanjan, M. M.: The Effectiveness of the Use of Mucinase in the Artificial Insemination of Cows. Sovet. zooteh., *8*, (1953): 86–88 (Anim. Breed. Abstr., *21*, [1953]: 351).

[2] Beshlebnov, A. V.: The Optimal Time of Inseminating Cows During Estrus. Probl. Zivotn., *2*, (1938): 73–88 (Anim. Breed. Abstr., *6*, [1938]: 291–292).

[3] Boyland, E.: The Composition of Bovine Cervical Mucins and Their Reactions with Oxidizing Agents. Biochem. J., *40*, (1946): 334–337.

[4] Brown, R. L.: Effect on Spermatozoa of Tissue Fluids Encountered in the Female Genital Tract. Am. J. Obst. and Gynec., *46*, (1943): 873–876.

[5] Calisti, V.: The Viscosity of the Mucus of the Genital Tract of the Cow in Relation to Temperature, Salts, pH, and Diffusing Factors. Zootec. e Vet., *3*, (1948): 131–138 (Anim. Breed. Abstr., *16*, [1948]: 221).

[6] Calisti, V., and Quacquarini, P.: The Fertility of Bull Semen Treated with Hyaluronidase. Zootec. e Vet., *7*, (1952): 239–243 (Anim. Breed. Abstr., *21*, [1953]: 48).

[7] Chang, M. C.: Development of Fertilizing Capacity of Rabbit Spermatozoa in the Uterus. Nature, *175*, (1955): 1035–1036.

[8] Colowick, S. P., and Kaplan, N. O.: Methods in Enzymology. vol. I. Academic Press, Inc., New York, 1955.

[9] Docton, F. L., Ferguson, L. C., Lazear, E. J., and Ely, F.: The Antigenicity of Bovine Spermatozoa. J. Dai. Sci., *35*, (1952): 706–709.

[10] Fiorentino, A., and Caretta, A.: The Results Obtained from the Addition of Crystalline Hyaluronidase to the Semen of *B. Taurus*. Zootec. e Vet., *6*, (1951): 449–453 (Anim. Breed. Abstr., *19*, [1951]: 465).

[11] Hawk, P. B., Oser, B. L., and Summerson, W. H.: Practical Physiological Chemistry. 13th ed. Blakiston Co., New York, 1954.

[12] Johnston, J. E., and Mixner, J. P.: Relationship of Hyaluronidase Concentration to Fertility of Dairy Bull Semen. J. Dai. Sci., *33*, (1950): 847–850.

[13] Kaemmerer, K., and Neumann, H. G.: Reducing Substances in the Cervical Mucus of Cattle. Ztschr. tierzärztl. Zucht. Biol., *58*, (1950): 416–421 (Anim. Breed. Abstr., *19*, [1951]: 59).

[14] Kirillov, V. S.: New Principles of the Organizations and Technique of Mating of Cows. Probl. Zivotn., *3*, (1937): 34–42 (Anim. Breed. Abstr., *5*, [1937]: 278).

[15] Kurzrok, R., Wilson, L., and Birnberg, C.: Follicular Fluid. Its Possible Role in Human Fertility and Infertility. Fert. and Ster., *4*, (1953): 479–492.

[16] Laing, J. A.: Observations on the Survival Time of the Spermatozoa in the Genital Tract of the Cow and Its Relation to Fertility. J. Agric. Sci., *35*, (1945): 72–83.

[17] Lindahl, P. E., and Kihlstrom, J. E.: An Antiagglutinic Factor in Mammalian Sperm Plasm. Fert. and Ster., *5*, (1954): 241–255.

[18] Lipphardt, E. M., and Pommerenke, W. T.: Effect of Addition of Spermatozoa on the Sugar Content of Cervical Mucus. Am. J. Obst. and Gynec., *59*, (1950): 918–920.

[19] Lutwak-Mann, C.: Note on the Chemical Composition of Bovine Follicular Fluid. J. Agric. Sci., *44*, (1954): 477–480.

[20] Matsunaga, E., and Itoh, S.: Intra-Uterine Selection by the ABO Incompatibility of Mother and Foetus. II. Proc. Japanese Acad., *29*, (1953): 529–531 (Biol. Abstr., *28*, [1954]: 2359).

[21] Matsunaga, E., and Itoh, S.: Intra-Uterine Selection by the ABO Incompatibility of Mother and Foetus. III. Differential Natality of Compatible and Incompatible Matings. Proc. Japanese Acad., *30*, (1954): 677–682 (Biol. Abstr., *30*, [1956]: 454).

[22] McLean, D., and Rowlands, I. W.: Role of Hyaluronidase in Fertilization. Nature, *150*, (1942): 627–628.

[23] Nelson, N.: A Photometric Adaptation of the Somogyi Method for the Determination of Glucose. J. Biol. Chem., *153*, (1944): 375–380.

[24] Olds, D., and VanDemark, N. L.: The Composition of Luminal Fluids in Bovine Female Genitalia. Fert. and Ster. (in press).

[25] Parsutin, G. V.: Spermatozoal Viability in the Mare's Uterus. Konevodestvo, *23*, (1952): 12–19 (Anim. Breed. Abstr., *21*, [1953]: 25).

[26] Perloff, W. H., Schultz, J., Farris, E. J., and Bolin, H.: Some Aspects of the Chemical Nature of Human Ovarian Follicular Fluid. Fert. and Ster., *6*, (1955): 11–16.

[27] Ross, V., Miller, E. G., Jr., and Kurzrok, R.: Metabolism of Human Sperm. Endocrinology, *28*, (1941): 885–893.

[28] Rumjancev, N. V., and Flegmatov, N. A.: On the Influence of Secretions of Female Genital Organs on Spermatozoa. Anim. Breed. Abstr., *6*, (1938): 254–255.

[29] Sallman, B., and Birkeland, J. M.: Interrelationships of Spermatozoa Count, Hyaluronidase Titer and Fertilization. Am. J. Physiol., *152*, (1948): 271–279.

[30] Sjostedt, S., Grubb, R., and Linell, F.: Blood Group Incompatibility in Abortion and Sterility. Acta Path. et Microbiol. Scand., *28*, (1951): 375–387 (Biol. Abstr., *26*, [1952]: 148).

[31] Sokolovskaja, I. I.: The Effect of Semen on the Mucin of the Animal Organism. Anim. Breed. Abstr., *18*, (1950): 172.

[32] Sokolovskaja, I. I., Rodin, I. I., Smirnov-Ugrjumov, D. V., and Tjupic, M. M.: The Use of Mucinase for Increasing Fertilization of Cows. Sovet. Zootch., *1950*, (1950): 65–74 (Anim. Breed. Abstr., *18*, [1950]: 404).

[33] Somogyi, M.: A New Reagent for the Determination of Sugars. J. Biol. Chem., *160*, (1945): 61–68.

[34] Vandeplassche, M., and Paredis, F.: Preservation of the Fertilizing Capacity of Bull Semen in the Genital Tract of the Cow. Nature, *162*, (1948): 813.

[35] Vandeplassche, M., and Paredis, F.: How Long Does Bull Sperm Maintain Its Fertilizing Capacity in the Genital Tract of the Cow? Tijdschr. Diergeneesk., *74*, (1949): 831–838 (Anim. Breed. Abstr., *19*, [1951]: 63).

Weight Gain in Phenothiazine-Treated Calves

GEO. E. CAUTHEN, D.V.M.

Angleton, Texas

IT IS generally agreed that most of the economic loss caused by the gastrointestinal parasites of cattle occurs in animals under 2 years of age. Investigations in the Coastal Prairie area of Texas have indicated that the majority of the 6-month-old beef calves have a worm eggs per gram of feces (e.p.g.) count of 100 to 300. This indicates a light to moderate subclinical degree of infection. Reports [1-4] of treating moderate to heavy subclinical infections in yearling and 2-year-old cattle show increased weight gain in treated animals compared to the controls. Turk *et al.*,[5] treating lightly infected, unweaned beef calves with 12.5 Gm. of phenothiazine per 100 lb. of live weight every 21 days during the last three to three and one-half months before weaning, reported that the rate of gain of the controls was equal to that of treated calves.

The general practice in much of the Coastal Prairie area of Texas is to sell calves at weaning. Any program that would reduce the worm population in unweaned calves might result in a heavier calf at the time of weaning or increased weight gain during the early postweaning period.

From Texas Substation 3, Angleton. Technical article 2653, published with the approval of the director of the Texas Agricultural Experiment Station, College Station.

The authors gratefully acknowledged the cooperation of the Hutching Farm, Sugarland Industries, and the Texas Prison System in providing the experimental sites and livestock necessary for the conduct of a part of these tests.

MATERIALS AND METHODS

Hereford, Brahman, and mixed Brahman-Hereford calves were used and the tests were conducted at the Texas Substation at Angleton and at five farms in the area. The one experiment at the Station and the nine on the five farms extended over a period of seven years. Some of the separate tests involved four different groups of calves, three of which were treated at different times in relation to time of weaning. In general, calves were weaned when 7 to 8 months old.

The phenothiazine used was green N.F. grade made into a drench, using bentonite as a wetting agent. Only one dose, varying from 60 to 96 Gm., was given to each calf.

For reporting the results, the calves were allotted to two large groups: calves with heavy subclinical and those with moderate subclinical parasitism. Calves with moderate subclinical infection were divided into: (1) preweaning treatment, test terminated at weaning; (2) preweaning treatment, test terminated after weaning; (3) treated at time of weaning; and (4) postweaning treatment.

TABLE 1—Heavy Subclinical Parasitism in Hereford Calves (Farm 1) Given Preweaning Treatment with Test Terminated After Weaning (1950)

Treatment						
No. of calves	Wt. (lb.)	Phenothiazine (Gm.)	Relation to weaning	Test period (da.)	Gain (lb.)	e.p.g.
14	280	0	20 to 50* BW†	97	−11	767
14	281	54	20 to 50* BW	97	+19	1,315
16	284	68	20 to 50* BW	97	+14	804
15	286	82	20 to 50* BW	97	+24	845
14	283	96	20 to 50* BW	97	+32	988

* Older calves were weaned 20 days after treatment; younger calves 50 days after treatment.
† BW = before weaning.

RESULTS

The e.p.g. counts are given as a means of aiding in determining the degree of parasitism. Counts less than 100 are considered to indicate light subclinical parasitism, 150 to 400 counts are moderate subclinical, and 600 to 1,000 counts are heavy subclinical parasitism for calves in this area.

Heavy Subclinical Parasitism.—The calves of this experiment (table 1) showed signs of clinical parasitism before termination of the test. The treated groups gained an average of 22 lb.; the controls lost 11 lb.

TABLE 2—Moderate Subclinical Parasitism in Calves Given Preweaning Treatment with Test Terminated at Weaning

No. of calves	Wt. (lb.)	Treatment: Phenothiazine (Gm.)	Relation to weaning	Termination of test	Gain (lb.)	e.p.g.
1949–1952—HEREFORD CALVES—EXPERIMENT STATION						
23	382	0	60 da. BW*	At weaning	46	176
22	382	60	60 da. BW	At weaning	46	218
1952—BRAHMAN—FARM 4						
74	286	0	68 da. BW	At weaning	105	48
74	286	60	68 da. BW	At weaning	102	29
1952—HEREFORD—FARM 3						
35	385	0	60 da. BW	At weaning	78	74
35	387	60	60 da. BW	At weaning	73	107
1952—BRAHMAN-HEREFORD—FARM 5						
12	254	0	62 da. BW	At weaning	83	412
13	274	60	62 da. BW	At weaning	85	446
Total						
144	323	0	.....	.....	87	.....
144	324	60	.....	.....	85	.....

* BW = before weaning.

Moderate Subclinical Parasitism. — In comparing the preweaning weights (table 2), control calves gained an average of 2 lb. more than those treated. In the experiments of preweaning treatment for postweaning gain (table 3), the treated calves gained more favorably than the control calves in six of the eight tests. The average gain of the treated calves in all eight tests was 5 lb. more than that of the controls. Calves treated at time of weaning (table 4) gained less than controls in three out of four tests. The controls averaged 4 lb. more than the treated calves. Of five postweaning experiments (table 5), treated calves of four gained less than untreated

TABLE 3—Moderate Subclinical Parasitism in Calves Given Preweaning Treatment with Test Terminated After Weaning

No. of calves	Wt. (lb.)	Treatment: Phenothiazine (Gm.)	Relation to weaning	Test period (da.)	Gain (lb.)	e.p.g.
1952—HEREFORD—FARM 3						
16*	386	0	60 da. BW‡	170 (110)‡	99	91
17*	384	60	60 da. BW‡	170 (110)	107	162
16*	386	0	60 BW and AW‡	170 (110)	99	91
16*	386	60	60 BW and AW‡	170 (110)	111	93
1953—BRAHMAN HEIFERS—FARM 4						
21	385	0	14 da. BW	117 (103)	−2	379
21	388	60	14 da. BW	117 (103)	3	528
1953—BRAHMAN STEERS—FARM 4						
21	406	0	14 da. BW	117 (103)	6	623
23	418	60	14 da. BW	117 (103)	14	315
1954—BRAHMAN HEIFERS—FARM 4						
18	386	0	14 BW and 90 AW	170 (156)	−15	238§
18	388	60	14 BW and 90 AW	170 (156)	− 9	238§
1954—BRAHMAN STEERS—FARM 4						
22	402	0	14 BW and 90 AW	170 (156)	22	238§
20	398	60	14 BW and 90 AW	170 (156)	4	238§
1954—BRAHMAN HEIFERS—FARM 4						
18†	385	0	28 da. BW	170 (142)	−15	238§
18	363	60	28 da. BW	170 (142)	− 7	238§
1954—BRAHMAN STEERS—FARM 4						
22†	402	0	28 da. BW	170 (142)	22	238§
16	392	60	28 da. BW	170 (142)	12	238§
Total						
154	393	0	.....	.....	24	.....
149	391	60	.....	.....	29	.....

* Served as treated group in test (table 2) of treating 60 days before weaning. † Served as untreated controls in concurrent test involving treatments at various times in relation to time of weaning. ‡ BW = before weaning, AW = after weaning. § Eggs per gram count of a composite fecal sample. ‖ Numbers in parentheses show days after weaning before weighed.

TABLE 4—Moderate Subclinical Parasitism in Calves Treated at Time of Weaning

No. of calves	Wt. (lb.)	Treatment: Phenothiazine (Gm.)	Relation to weaning	Test period (da.)	Gain (lb.)	e.p.g.
1952—HEREFORD CALVES—FARM 3						
16*	463	0	At weaning	110	23	110
13*	464	60	At weaning	110	17	239
1953—BRAHMAN STEERS—FARM 4						
21†	406	0	At weaning	117 (103)	6	618
22	409	60	At weaning	117 (103)	6	687
1953—BRAHMAN HEIFERS—FARM 4						
21†	385	0	At weaning	117 (103)	−2	379
20	389	60	At weaning	117 (103)	0	467
1956—BRAHMAN STEERS—FARM 4						
32	369	0	At weaning	121	61	.....
95	371	65	At weaning	121	51	.....
1956—BRAHMAN HEIFERS—FARM 4						
31	345	0	At weaning	121	39	.....
98	345	65	At weaning	121	34	.....
Total						
121	383	0	At weaning	.....	30	.....
248	384	60 to 65	At weaning	.....	26	.....

* Served as untreated controls in test (table 2) of treating 60 days before weaning. † Served as untreated controls in concurrent tests involving treatments at various times in relation to time of weaning.

calves. The average gain was 1 lb. more in the controls than in the treated.

Necropsy records (table 6) of Station calves (table 2) indicate that treatment reduced the worm population even when necropsy was delayed 60 days after treatment. The average number of worms per calf, of species other than Cooperia, found in the treated calves was 61; in the controls, it was 211. The average number of Cooperia found in the treated calves was 5,212; in the controls, it was 6,097.

DISCUSSION

In the reports of previous workers,[2-4] the yearlings and 2-year-old cattle, with a moderate subclinical degree of parasitism, treated with phenothiazine gained more than the untreated controls. Younger cattle should respond equally as well to treatment. However, in the experiments reported here, calves with moderate subclinical parasitism treated with phenothiazine gained less than controls in three out of four group experiments.

TABLE 5—Moderate Subclinical Parasitism in Calves Given Postweaning Treatment

No. of calves	Wt. (lb.)	Treatment: Phenothiazine (Gm.)	Relation to weaning	Test period (da.)	Gain (lb.)	e.p.g.
1950—BRAHMAN HEIFERS—FARM 1						
41	349	0	60 da. AW†	66	3.4	264
40	349	90	60 da. AW	66	−1.6	267
1951—BRAHMAN HEIFERS—FARM 1						
26	343	0	30 da. AW	89	8.0	220
13	340	90	30 da. AW	89	4.0	201
1954—BRAHMAN HEIFERS—FARM 4						
18*	386	0	90 da. AW	170 (142)	−15.0	238‡
16	375	60	90 da. AW	170 (142)	− 5.0	238‡
1954—BRAHMAN STEERS—FARM 4						
22*	402	0	90 da. AW	170 (142)	23.0	238‡
21	392	60	90 da. AW	170 (142)	18.0	238‡
1956—BRAHMAN HEIFERS—FARM 4						
38	353	0	67 da. AW	87	28.0	235
17	366	65	67 da. AW	87	27.0	208
Total						
145	365	0	.....	.....	8.0	.....
107	363	60 to 90	.....	.....	7.0	.....

* Served as untreated control in concurrent tests. † AW = after weaning. ‡ Eggs per gram count of composite fecal sample.

TABLE 6—Necropsy Records of Experiment Station Calves 60 Days After Treatment

Year	No. of calves	Phenothiazine (Gm.)	Haemonchus	Ostertagia	Trichostrongylus	Cooperia	Bunostomum	Oesophagostomum
1949	4	0	133	725	12	10,412	72	143
	3	60	15	266	0	9,733	36	31
1950	3	0	138	167	50	1,783	18	16
	3	60	18	50	0	700	7	1

The failure to obtain greater weight gain in the treated calves may be due to one of several factors: (1) The phenothiazine (N.F. grade) may have been toxic to the extent of canceling any benefits, so that the weight gain of the treated was at the same level as that of the controls. (2) The resistance of the calves to the worm parasites may have been equal to the adverse effect of the worms on the calves, resulting in an equilibrium of the host-parasite relationship and no benefit from removal of the worms. (3) It may have been due to a combination of the above factors.

SUMMARY

In ten separate experiments, over a seven-year period, Hereford, Brahman, and mixed Hereford-Brahman calves (707 treated, 578 controls) were given single doses of phenothiazine (60 to 96 Gm.) at various times from 68 days before to 90 days after weaning.

In the one experiment involving calves with heavy subclinical parasitism, the treated calves gained an average of 22 lb.; controls lost 11 lb.

In only one out of four groups of calves with moderate subclinical parasitism was there a greater weight gain (5 lb.) in the treated calves. The treated calves in the other three groups averaged 3 lb. less than the controls.

References

[1] Stewart, W. A., and Crofton, H. D.: Parasitic Gastritis in Cattle. Vet. Rec., *63*, (1941): 619–621.

[2] E. I. duPont de Nemours and Co.: Economics of Controlling Parasites with Phenothiazine. E. I. duPont Tech. Bull. 6, (1953): 2–4, 20, 27–28.

[3] Boughton, D. C., and Bacon, R. J.: Phenothiazine in the Control of Internal Parasites of Beef Cattle. New Information About Internal Parasites—Review of Livestock Parasites. E. I. duPont Co., *2*, (1954): 1–6.

[4] Porter, D. A., Simms, B. T., and Cauthen, G. E.: Field Test with Phenothiazine as an Anthelmintic in Cattle. J.A.V.M.A., *99*, (1941): 272–278.

[5] Turk, R. D., Jones, J. H., and Crouch, E. K.: Phenothiazine in Unweaned Calves. Am. J. Vet. Res., *15*, (1954): 224.

Low Level Phenothiazine Administration to Weaned Beef Calves

GEORGE E. CAUTHEN, D.V.M.

Angleton, Texas

THE FREE-CHOICE administration of phenothiazine in a salt and mineral mixture has, in general, been successful in controlling the gastrointestinal parasites of sheep. Foster,[1] in 1953, reviewed the literature dealing with a similar procedure in cattle and stated that the employment of 5 to 10 per cent of phenothiazine in salt or mixed minerals had worked efficiently in some instances but had failed in others. Dennis *et al.*, in 1955, reviewed the literature dealing with low level administration of phenothiazine to cattle by way of mineral and feed.

Herlich and Porter[3] reported that free-choice administration of a phenothiazine-mineral mixture to Jersey calves effectively controlled the level of parasitic infection of Haemonchus and Oesophagostomum but showed no significant effect on Ostertagia, Trichostrongylus, Cooperia, and Nematodirus. Riedel *et al.*[4] reported increased weight gains and reduced parasitism in dairy calves when given low levels of phenothiazine in the feed. Shelton *et al.*,[5] using dairy calves, reported that calves with a moderate degree of infection given phenothiazine in their feed daily made slightly better gains than calves not receiving phenothiazine. The amount of parasitism acquired by susceptible young calves on pasture was considerably less when all older animals on that pasture were receiving low level phenothiazine in the feed.

The studies reported here were undertaken to further evaluate the effect of low level phenothiazine when administered to weaned beef calves.

MATERIALS AND METHODS

Calves from three different sources were used. The first two experiments were conducted at the same farm using Brahman calves. The last two experiments were conducted at the Station using Station calves (¾ Hereford and ¼ Brahman) and grade Hereford calves from a farm in the area. The phenothiazine used was N.F. grade.

Experiment 1 (1950).—Approximately 60 days after the calves were weaned (when 7 to 8 months old), they were given 90 Gm. of phenothiazine and allotted to two groups. Those in one group (medicated) were given an average of 1 lb. of a mixture of 92.0 per cent cottonseed meal, 8.0 per cent mineral (salt and bone meal), and 0.25 per cent phenothiazine. This was mixed with sorghum silage and provided approximately 1 Gm. of phenothiazine per animal per day. The second group (controls) received the same ration without phenothiazine.

Experiment 2 (1951).—This test was similar to experiment 1 except that the calves were given 90 Gm. of phenothiazine 30 days after weaning and the medicated calves received an average of 2 Gm. of phenothiazine daily.

Experiment 3 (1951).—Station calves were weaned when 6 to 7 months of age and immediately allotted to two groups. These calves were not given an initial dose of phenothiazine. The medicated calves were given 2 Gm. of phenothiazine daily in a commercial grain ration. The controls received the same ration without phenothiazine.

Experiment 4 (1952).—This test was similar to experiment 3 except that the calves were weaned at 7 to 8 months of age.

All calves in the four tests were weighed and worm eggs per gram of feces (e.p.g.) counts were made at various times during the test.

RESULTS

The results of the weight gain and e.p.g. counts for the four experiments are given (table 1).

In experiments 1 and 2, an initial dose of 90 Gm. of phenothiazine was given. In the first experiment, there was no difference in the weight gain of the 40 calves receiving 1 Gm. of phenothiazine daily for 122 days and the 41 control calves. In the second experiment, the 8 calves receiving 2 Gm. of phenothiazine daily gained an average of 20 lb. more than the 8 control calves during the 157-day test period.

In experiments 3 and 4, no initial dose of phenothiazine was given. The 5 calves of the third experiment received 2 Gm. of phenothiazine daily and gained an average of 60 lb. more than the 6 control calves

From Texas Substation 3, Angleton. Technical article 2482, published with the approval of the director of the Texas Agricultural Experiment Station, College Station.

The authors gratefully acknowledge the cooperation of the Texas Prison System in providing pastures, cattle, and aid in handling of the cattle at the Darrington State Farm.

TABLE 1—Weight Gains and Fecal Worm Egg (e.p.g.) Counts of Control Calves and Calves Receiving 1 and 2 Gm. of Phenothiazine Daily

No. of calves	Age (mo.)	Gm. of phenothiazine daily	Duration (da.)	Wt. at beginning	Gain (lb.)	Worm eggs per gram of feces		
						Max. e.p.g.	No. counts	Av. e.p.g.
EXPERIMENT 1—BRAHMAN CALVES, 1950								
41	9 to 10	0	122	349	11	285	2	161
40	9 to 10	1	122	349	11	267	2	127
EXPERIMENT 2—BRAHMAN CALVES, 1951								
8	8 to 9	0	157	331	45	314	4	205
8	8 to 9	2	157	338	70	212	4	142
EXPERIMENT 3—HEREFORD CALVES, 1951								
6	6 to 7	0	179	267	98	1,262	5	325
5	6 to 7	2	179	267	158	867	5	144
EXPERIMENT 4—HEREFORD-BRAHMAN CALVES, 1952								
4	7 to 8	0	88	530	−15	267	3	219
4	7 to 8	2	88	525	20	184	3	140

during the 179-day test period. In the fourth experiment, the 4 calves which received 2 Gm. of phenothiazine daily gained an average of 25 lb., whereas the 4 controls lost an average of 15 lb. during the 88-day test.

The e.p.g. counts of the calves of experiments 1, 2, and 4 indicated a moderate subclinical degree of parasitism; of experiment 3, a moderate to heavy subclinical infection.

The effect of low level phenothiazine administration on the e.p.g. count and the average e.p.g. count (not including the first which was made at the beginning of the test) are also shown. The average e.p.g. counts of the control calves were 26, 44, 125, and 56 per cent more than those of the respective medicated groups.

SUMMARY

In 1950, 40 untreated weaned calves with moderate subclinical parasitism gained as well but had a 26 per cent greater fecal worm egg count than 41 similar calves which received 1 Gm. of phenothiazine in their feed for 122 days.

In 1951 and 1952, in three trials, 18 untreated weaned calves with moderate or moderate to heavy parasitism gained 25 to 60 lb. less and had 44 to 125 per cent higher fecal worm egg counts than 17 calves which were fed 2 Gm. of phenothiazine daily for 88 to 179 days.

References

[1] Foster, A. O.: Free-Choice Phenothiazine for Cattle, Vet. Med., *48*, (1953): 208.

[2] Dennis, W. R., Swanson, L. E., and Stone, W. M.: Experimental Feeding of Low Level Phenothiazine to Florida Cattle, Vet. Med., *50*, (1955): 379.

[3] Herlich, H., and Porter, D. A.: Control of Internal Parasites of Cattle by Free-Choice Administration of Phenothiazine, Vet. Med., *49*, (1954): 103.

[4] Riedel, B. B., Owen, J., and McCrary, H. F.: Phenothiazine as Parasite Control for Dairy Calves, Mississippi Farm Res., *18*, (1955): 2.

[5] Shelton, G. C., Rodabaugh, D. E., and Elder, C.: Some Results of Phenothiazine as a Means of Controlling Gastrointestinal Parasites of Calves, Vet. Med., *51*, (1956): 551.

Elementary Bodies of Sheep Pox

M. ABDUSSALAM, Ph.D., L.V.P.*

College Park, Maryland

ELEMENTARY BODIES of sheep pox virus were among the first to be seen with the ordinary light microscope (Borrel, 1903)[3] but their morphology does not appear to have been studied either with recent methods of staining or with the electron microscope. We have studied a Pakistani strain of the virus by these methods and found it to be morphologically allied to, but smaller than, the virus of contagious ecthyma (orf).

MATERIAL AND METHODS

The strain of the virus studied was isolated in 1952 at Lahore, West Pakistan, from a fat-tailed sheep suffering from the generalized form of the natural disease. Since then, it has been maintained in lambs by skin-to-skin passage, often interrupted with storage in the refrigerator (0 to 5 C.). Immunological and cross-protection studies of this strain against an Algerian strain of sheep pox virus † showed that the two viruses are identical.

Elementary bodies were isolated from experimental skin lesions in lambs by differential centrifugation as is done for vaccinia and contagious ecthyma (Parker and Rivers, 1935; Abdussalam and Cosslett, 1957),[5,9] Distilled water, instead of buffer solution, was used for washing and storing the virus. The final suspension was comparable in opacity with Brown's tube No. 5 and was infective for lambs by intradermal inoculation in a dilution of 1:32,000.

For staining, smears of the foregoing suspension as well as of skin lesions were used. The skin smears were dried in the air and kept undisturbed on edge in distilled water for five to ten minutes. They were then dried again and fixed for two minutes in methyl alcohol before staining.

For electron micrography, the concentrated elementary body suspension was fixed and inactivated by adding five per cent formalin. Some of the fixed virus was spun down in an angle centrifuge and resuspended in distilled water immediately before study. The suspensions were diluted in volumes of distilled water and placed on paralodion film supports which had been prepared on the previous day. The excess fluid was removed with a capillary pipette and the films dried. Some preparations were shadowed with gold (Williams and Wyckoff, 1946)[12] at arc tangent 2/10 and examined under the RCA electron microscope, type E.M.U. Most of the electron micrographs were taken at a magnification of 18,000 and were subsequently enlarged to 40,000 for measurement and study.

Some of the remaining suspension was smeared and stained by Paschen's method. It showed numerous elementary bodies indistinguishable from those seen in smears from sheep pox lesions.

STAINING CHARACTERS

Sheep pox elementary bodies in purified suspension stained well with Paschen's, Gutstein's (1937; methyl violet),[5] and Morosow's (1926)[7] methods after fixation with methyl alcohol for two minutes. Staining with the first two methods was less intense, often weak, if fixation was omitted. Only weak staining was obtained with Giemsa, crystal violet (1%), Herzberg's Victoria blue, and carbol fuchsin. They did not stain with methylene blue, dilute carbol fuchsin, or eosin.

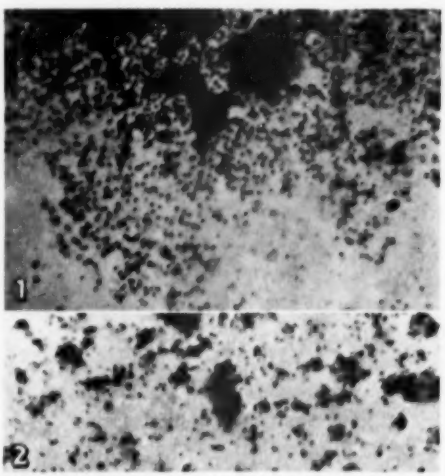


Fig. 1—Elementary bodies of sheep pox in smear of early vesicular lesion on skin of lamb. Paschen's stain. x 1,200.

Fig. 2—Agglutinated and dispersed elementary bodies in old suspension of sheep pox virus. Paschen's stain. x 1,000.

* The section on electron micrography was written in collaboration with Reginald L. Reagan, College Park, Md. Dr. Abdussalam's present address is: College of Animal Husbandry, Lahore, Pakistan.

The author thanks Drs. R. J. Byrne, College Park, Md., and G. R. Spencer, Pullman, Wash., for facilities and interest in this work.

† This virus was obtained from Dr. Rafiyi of Hessarek, Iran.

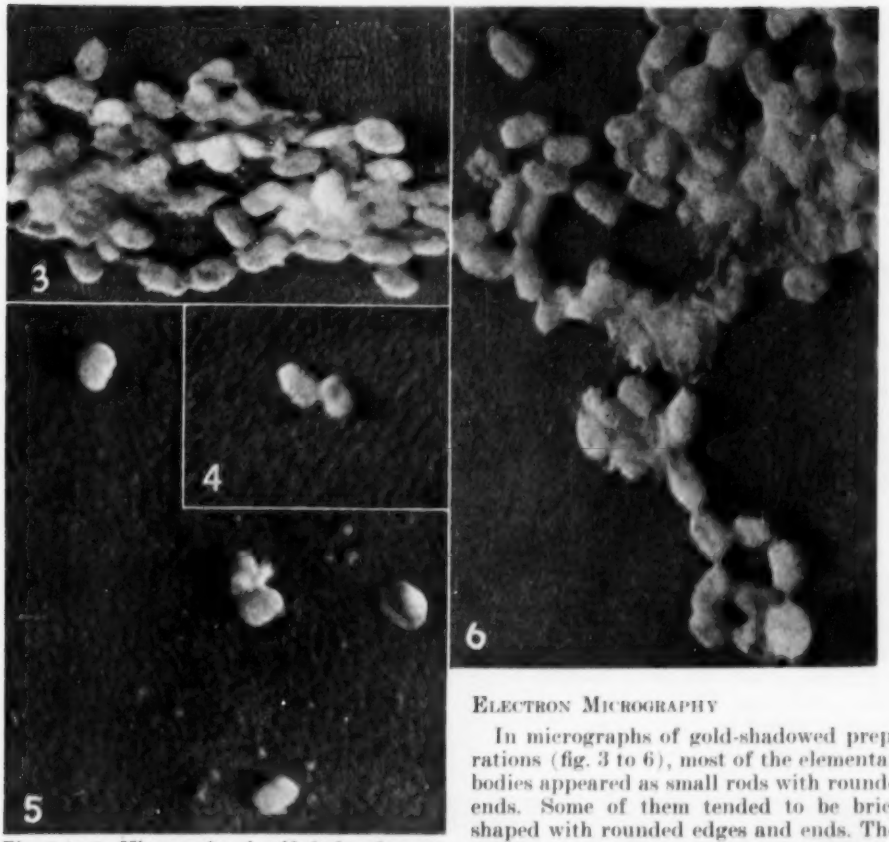


Fig. 3 to 6—Micrographs of gold-shadowed smear of sheep pox elementary bodies. x 40,000.

In smears of skin lesions before pustulation, numerous elementary bodies could be seen after staining by Paschen's method (fig. 1, 2). With Gutstein's method (long as well as short procedure), the virus stained less intensely than in smears of purified suspension. In tissue smears stained with Morosow's method, nonviral material interfered with the identification of elementary bodies.

In Paschen-stained smears, the elementary bodies appeared as bright red, small round bodies disposed singly, in pairs, small chains, or clusters. Thick clusters were dark red and somewhat homogenous —outlines of individual bodies could not be distinguished.

Electron Micrography

In micrographs of gold-shadowed preparations (fig. 3 to 6), most of the elementary bodies appeared as small rods with rounded ends. Some of them tended to be brick-shaped with rounded edges and ends. They dried with a greater degree of collapse on the paralodion film than the elementary bodies of contagious ovine ecthyma virus (Abdussalam and Cosslett, 1957),[1] even though fixation in formalin must have hardened them to some extent. Closely placed elementary bodies were connected by strands, which indicates that they must have a tenacious surface layer.

The surface of many elementary bodies showed a central depression which was elongated and disposed in a direction parallel to the long axis in some of them. The surface of many elementary bodies, especially those that appeared to be disintegrating, showed small nodular protuberances but they were not disposed in a regular pattern as in the rabbit papilloma virus (Williams, 1953).[11] Small, roundish particles, probably derived from the virus, were seen near disintegrating elementary bodies (fig. 3, 5, 6).

TABLE 1—Comparison of Dimensions of Sheep Pox Virus with Related Viruses (All Measurements in Millimicrons)

Virus	No. of elementary bodies measured	Mean dimensions			Reference
		Length (a)	Breadth (b)	Axial ratio Q = a/b	
Molluscum contagiosum	150	255 ± 61.3	178 ± 41.8	1.45 ± 0.143	Ruska and Kausche (1943)[10]
Ectromelia	90	232 ± 24.6	172 ± 9.6	1.34 ± 0.024	Ruska and Kausche (1943)[10]
Rabbit myxoma	81	287 ± 20.7	233 ± 19.0	1.23 ± 0.122	Ruska and Kausche (1943)[10]
Fowlpox	100	322 ± 90.2	264 ± 73.9	1.22	Boswell (1948)[4]
Canary pox	75	311 ± 26.1	263 ± 22.7	1.14 ± 0.108	Ruska and Kausche (1943)[10]
Vaccinia	70	262 ± 28.2	209 ± 23.9	1.25 ± 0.124	Ruska and Kausche (1943)[10]
Swine pox	24	323 ± 68.2	240 ± 46.6	1.35 ± 0.31	Blakemore and Abdussalam (1956)[2]
Contagious ecthyma	206	252 ± 22.5	158 ± 17.7	1.60 ± 0.16	Abdussalam & Cosslett (1957)[1]
Sheep pox	160	194 ± 16.08	115 ± 10.73	1.76 ± 0.176	.....

Micrographs of unshadowed elementary bodies (fig. 7, 8) revealed some evidence of internal structure. Many of them showed two dense areas of higher electron-absorbing power in the subpolar regions, one of which was frequently more prominent than the other. The dense spot seen in the center of vaccinia, fowlpox, and rabbit myxoma viruses was not present. Indeed, some of the elementary bodies showed a less dense and clearer center.

Dimensions.—The elementary bodies measured 150 to 250 mμ in length, with an average of 194 ± 16.08 mμ. They were 88 to 138 mμ broad, the mean breadth being 115 ± 10.73 mμ. The axial ratio (length/breadth) varied between 1.3 and 2.71 with an average of 1.76 ± 0.176.

DISCUSSION

In its staining characters, the sheep pox virus resembles other animal pox viruses, particularly that of contagious ecthyma. In this study, best staining was obtained with Paschen's method which can be used for quick laboratory diagnosis of the disease. This method does not differentiate sheep pox from contagious ecthyma or vaccinia and can not be applied after suppuration has set in, but in spite of these limitations it can be a valuable aid in clinical as well as experimental work.

In its morphology, as revealed by the electron microscope, sheep pox virus resembles other animal pox viruses in several respects; however, it is more elongated in shape. In shape and internal structure

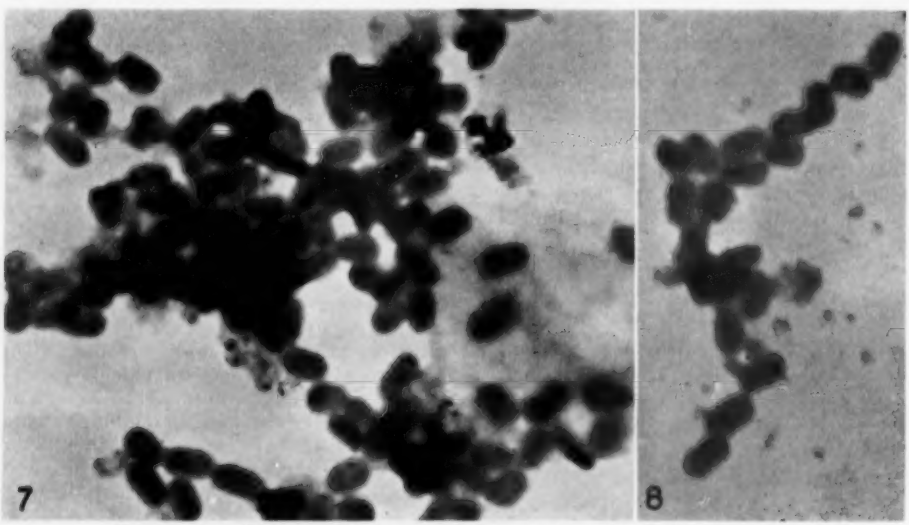


Fig. 7 and 8—Unshadowed smears of sheep pox elementary bodies. x 40,000.

(subpolar densities), it resembles the virus of contagious ecthyma but differs from it in being smaller in size and slightly longer. It also dries with a greater degree of collapse on a flat surface. Its dimensions are compared with those of related viruses (table 1). It will be seen that it is distinguishable from other members of the pox group.

The dimensions of the elementary bodies measured from the electron micrographs are somewhat smaller than those of the infective particles ascertained from filtration experiments. Levaditi, Bridré, and Krassnoff (1938)[6] calculated the approximate size of a virulent strain to be 170 to 260 mμ and that of a less virulent strain to be 250 to 380 mμ. It is noteworthy that their less virulent strain resembled the contagious ecthyma virus in size.

SUMMARY

Elementary bodies of the sheep pox virus were isolated by differential centrifugation from early skin lesions of lambs and studied for staining characters and morphology.

Paschen's method was found to be the best for staining purified elementary bodies as well as smears of skin lesions. Other methods found successful for staining the virus, but not as satisfactory as Paschen's method, were Morosow's and Gutstein's.

In electron micrographs, the virus was found to resemble other pox viruses but was smaller and more elongated. It appeared to have subpolar densities like the virus of contagious ecthyma. No central dense spot could be demonstrated.

Average dimensions were: length, 194 ± 16.08 mμ; breadth, 115 ± 10.73 mμ; and axial ratio (length/breadth), 1.76 ± 0.176.

References

[1] Abdussalam, M., and Cosslett, V. S.: Morphology of the Contagious Pustular Dermatitis Virus. J. Comp. Path. (1957): In press.

[2] Blakemore, F., and Abdussalam, M.: Morphology of the Elementary Bodies and Cell Inclusions in Swine Pox. J. Comp. Path., *66*, (1956): 373–377.

[3] Borrel, A.: Épithélioses Infectieuses et Épithéliomas. Ann. Inst. Pasteur, *17*, (1903): 81–122.

[4] Boswell, F. W.: Electron Microscope Studies of Virus Elementary Bodies. Brit. J. Exptl. Path., *28*, (1947): 253–260.

[5] Gutstein, M.: New Direct Staining Methods for Elementary Bodies. J. Path. Bact., *45*, (1937): 313.

[6] Levaditi, C., Bridré, J., and Krassnoff, D.: Dimensions approximatives du virus de la clavelée, déterminées par l'ultra-filtration. Compt. rend. Acad. d. sc. Paris, *206*, (1938): 953–954.

[7] Morosow, M. A.: Die Färbung der Paschenschen Körperchen durch Versilberung. Zentralbl. f. Bakt. I. (Orig.), *100*, (1926): 385.

[8] Nagler, F. P. O., and Rake, G.: The Use of the Electron Microscope in Diagnosis of Variola, Vaccinia and Varicella. J. Bact., *55*, (1948): 45.

[9] Parker, R. F., and Rivers, T. M.: Immunological and Chemical Investigations of Vaccine Virus. I. Preparation of Elementary Bodies of Vaccinia. J. Exptl. Med., *62*, (1935): 65–72.

[10] Ruska, H., and Kausche, G. A.: Ueber Form, Grössenverteilung und Struktur einiger Virus-Elementarkörper. Zentralbl. f. Bakt. I. (Orig.), *150*, (1943): 311–318.

[11] Williams, R. C.: The Shape and Sizes of Purified Viruses as Determined by Electron Microscopy. Cold Spring Harbour Symp. Quant. Biol., *18*, (1953): 185–195.

[12] Williams, R. C., and Wyckoff, R. W. G.: Applications of Metallic Shadow-Casting to Microscopy. J. Applied Physics., *17*, (1946): 23.

The Cultivation of Sheep Pox Virus on the Chorioallantoic Membrane of the Developing Chicken Embryo

M. S. SABBAN, D.V.M., M.V.Sc., Ph.D.

Alexandria, Egypt

SHEEP POX is widely spread among sheep in most countries of the Eastern Hemisphere; therefore, the production of seed virus from a cheap host for the preparation of the vaccine is of great value.

Hutyra and Marek [1] in their description of the different methods for the large-scale production of sheep pox lymph mentioned that the sheep was the animal used for such production. In all of the methods described by different workers [2-8] for the preparation of different types of sheep pox vaccine, the sheep was the animal used for the production of seed virus.

Rao,[9] in his study on the cultivation of sheep pox virus on the chorioallantoic membrane (c.a.m.) of the chicken embryo, recorded that after a few egg passages the virus was partially converted into vaccinia virus, as it succeeded in infecting the rabbit and calf and in cross-immunizing with the vaccinia virus. Blaxall [10] mentioned that sheep pox virus could be converted into vaccinia by passage through the goat, calf, and rabbit. Findlay [11] stated that before claims of conversions of sheep pox virus into vaccinia can be accepted, more extensive and carefully controlled experimental evidence is needed. Gins and Kunert [12] recorded that vaccinia virus protected sheep against natural sheep pox virus. In their trials to cultivate the sheep pox virus on the membrane of the developing chicken embryo, they recorded that the cultured virus failed to protect sheep against the natural sheep pox virus. Bennett *et al.* [13] demonstrated that sheep previously infected with vaccinia virus were fully susceptible to sheep pox virus. Ortenzi and Tibeco [14] stated that their attempts to cultivate sheep pox virus on the c.a.m. of 9-day chicken embryos were unsuccessful; however, during the first passage, there was an initial though transitory multiplication of elementary bodies. Chamsy,[15] in Iran, mentioned that trials to cultivate the sheep pox virus on the c.a.m. were not successful. In the work that follows, a trial was made to cultivate the sheep pox virus on the c.a.m. of the developing chicken embryo.

From the High Institute of Public Health, Hadra, Alexandria, Egypt.

MATERIALS AND METHODS

Technique.—Strains of three Egyptian sheep pox viruses were secured from widely separated outbreaks in Egypt. They were tried on young susceptible lambs and found to give typical lesions. One of these strains was found to be virulent and was called the "Cairo 8" strain. The Romanian strain was used for trial of cultivation in the egg and for challenge of immunity. Stocks, desiccated under vacuum, were prepared from the Egyptian Cairo 8 and from the Romanian strain. After being tested on susceptible lambs, they were stored at —20 C. The sheep used in this work were from the Breeding Experimental Farms belonging to the Ministry of Agriculture. Lambs, 3 to 4 months old, that had no history of previous exposure to, or vaccination against, sheep pox were used. Fertile hens' eggs were brought from the experimental poultry breeding farms. Twelve-day eggs were inoculated on the c.a.m. by the window method using hot paraffin for dropping the membrane and Scotch tape for covering the window. The technique employed for titrating the infected material on sheep has been described previously.[8]

EXPERIMENTAL RESULTS

Alternating Passage of the Sheep Pox Virus Between Sheep and Chicken Embryos.—Nakamura and Miyamoto,[16] in Japan, were successful in adapting a lapinized rinderpest virus for eggs by alternating passages between rabbits and eggs. Since direct serial passage of our sheep pox virus on the c.a.m. of eggs seems to be extremely difficult, alternating passages of this virus in embryonating eggs and sheep were attempted (table 1). This procedure was initiated with the Cairo 8 strain of Egyptian sheep pox virus. A 10 per cent suspension of infected sheep fascia was inoculated on the c.a.m. of 12-day eggs, each receiving 0.2 ml. After four days of incubation at 36 C., the injected membranes were collected from all viable embryos and ground with sterile saline to make a 10 per cent suspension. From this dilution, tenfold dilutions up to 10^{-6} were

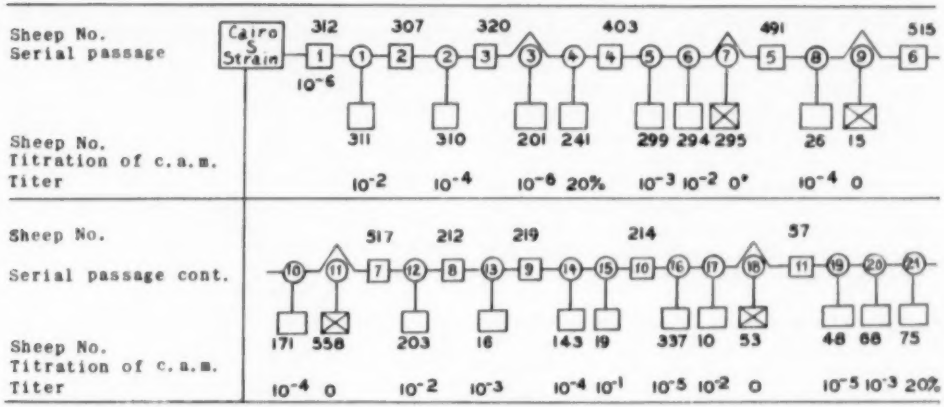


TABLE 1—Alternating Passages of the Sheep Pox Virus Between Sheep and Chicken Embryos

prepared. Each dilution was inoculated, 1.0 ml. intradermally in three areas, on the side of the belly of 1 sheep. In another sheep, the 10 per cent suspension was inoculated subcutaneously in different areas on the side of the belly. The first sheep was used for titrating the c.a.m. and the second was used for the transference of the virus.

The Egyptian Cairo S strain of sheep pox virus was successfully transferred on the c.a.m. using the alternating method for 21 passages. The original virus when titrated on sheep 312 reached 10^{-6}. When the virus was inoculated in eggs, the first alternating egg passage showed a marked drop in its amount as the titer was only 10^{-2} in sheep 311. The virus increased gradually in the second alternating egg passage and reached a titer of 10^{-4} in sheep 310. In the third alternating egg passage, the amount of virus in the membrane was equal to that of the original sheep virus as the titer was 10^{-6} in sheep 201. When the third alternating egg passage virus was inoculated in another group of eggs, the amount of virus dropped to a great extent, as it showed a positive reaction in sheep 241 at a dilution of 20 per cent. The virus was successfully cultivated on the c.a.m. for three direct passages. The c.a.m. collected

Fig. 1—Sheep 75 showing generalized pox lesions. This sheep was inoculated with the twenty-first alternating passage or the third direct chorioallantoic membrane passage of the Cairo S Egyptian sheep pox virus.

from the egg passages 7, 9, 11, and 18 when inoculated in sheep 295, 15, 558, and 53, respectively, did not cause any febrile or skin reactions. When these sheep were challenged with a known virus, they all showed typical febrile and skin reactions. This indicated that no virus had grown in these membrane passages. It has also been observed that by such egg passage the virus did not show any marked change in virulence toward sheep, as the third direct egg passage caused generalized infection in sheep 75 (fig. 1).

From these results, it could be concluded that the sheep pox virus was successfully cultivated in the egg and that the amount of virus obtained from the c.a.m. in some passages was nearly equal to that obtained from the sheep and, therefore, the egg could be used as a cheap host for the production of the seed virus for the preparation of the vaccine.

TABLE 2—Rate of Propagation of Sheep Pox Virus on the Chorioallantoic Membrane (c.a.m.) of the Developing Chicken Embryo

Days of incubation postinoculation	Sheep No.	Dilution of c.a.m.	Results Area 1	Area 2	Area 3	Titer
1	1168	1:5	+	+	+	10^{-4}
		1:10	+	+	+	
		1:100	+	+	−	
		1:1,000	+	−	−	
		1:10,000	−	−	−	
		1:100,000	−	−	−	
		1:1,000,000	−	−	−	
2	251	1:5	+	+	+	10^{-4}
		1:10	+	+	+	
		1:100	+	+	+	
		1:1,000	+	+	+	
		1:10,000	+	+	+	
		1:100,000	+	−	−	
		1:1,000,000	−	−	−	
3	271	1:5	+	+	+	10^{-4}
		1:10	+	+	+	
		1:100	+	+	+	
		1:1,000	+	+	+	
		1:10,000	+	+	+	
		1:100,000	−	−	−	
		1:1,000,000	−	−	−	
4	771	1:5	+	+	+	10^{-4}
		1:10	+	+	+	
		1:100	+	+	+	
		1:1,000	+	+	+	
		1:10,000	+	+	+	
		1:100,000	+	−	−	
		1:1,000,000	−	−	−	
5	557	1:5	+	+	+	10^{-3}
		1:10	+	+	+	
		1:100	+	+	+	
		1:1,000	+	+	+	
		1:10,000	−	−	−	
		1:100,000	−	−	−	
		1:1,000,000	−	−	−	
6	272	1:5	+	+	+	10^{-3}
		1:10	+	+	+	
		1:100	+	+	+	
		1:1,000	+	−	−	
		1:10,000	−	−	−	
		1:100,000	−	−	−	
		1:1,000,000	−	−	−	

+ = positive local reaction; − = negative local reaction.

TABLE 3—Distribution of Sheep Pox Virus in the Developing Chicken Embryo

Part of chicken embryo	Sheep No.	Dilutions	Results Area 1	Area 2	Area 3	Titer
Chorio-allantoic mem-brane	1102	1:5	+	+	+	10^{-4}
		1:10	+	+	+	
		1:100	+	+	+	
		1:1,000	+	+	+	
		1:10,000	+	+	−	
		1:100,000	+	−	−	
		1:1,000,000	−	−	−	
Yolk sac	1189	1:2.5	+	+	+	10^{-2}
		1:10	+	+	+	
		1:100	+	+	−	
		1:1,000	+	−	−	
		1:10,000	−	−	−	
		1:100,000	−	−	−	
		1:1,000,000	−	−	−	
Embryo	1190	1:2.5	+	+	−	10^{-1}
		1:10	+	+	−	
		1:100	−	−	−	
		1:1,000	−	−	−	
		1:10,000	−	−	−	
		1:100,000	−	−	−	
		1:1,000,000	−	−	−	
Chorio-allan-toic fluid	1191	Undiluted	+	+	+	10^{-1}
		1:10	+	+	−	
		1:100	−	−	−	
		1:1,000	−	−	−	
		1:10,000	−	−	−	
		1:100,000	−	−	−	
		1:1,000,000	−	−	−	

+ = positive local reaction; − = negative local reaction.

Studies on the Cultivation of Sheep Pox Virus on the Chorioallantoic Membrane of the Developing Chicken Embryo.—In the previous experiments on the cultivation of the virus on the c.a.m. of the chicken embryos, 12-day eggs that were incubated for four days after inoculation at 36 C. were chosen arbitrarily. To determine the optimal day of transfer, the best age of eggs, the suitable temperature, and the part of egg in which the maximal viral concentration is present, the following experiments were carried out.

Rate of Propagation of the Virus on the Chorioallantoic Membrane.—One hundred and twenty eggs, incubated for 12 days prior to inoculation, were injected on the c.a.m. with a 10 per cent suspension of fascia infected with the sixth sheep alternating passage of the Egyptian sheep pox virus. Each day, 20 membranes from living embryos were harvested beginning with the first day after inoculation and ending with the sixth. Immediately after removal, suspensions were made from the membranes and titrated in sheep (table 2).

After 24 hours of incubation, there was growth of virus in the c.a.m. as the titer reached 10^{-2}. After 48 hours, the amount of virus reached its peak and the titer reached 10^{-4}. After three and four days of incubation, the virus in the membrane remained high as the titer reached 10^{-4}. After five days, the titer of virus started to drop and reached 10^{-3} and, after six days of incubation, the virus dropped more and reached 10^{-2}. From the results of this experiment, it could be seen that the optimal day of transfer of infected c.a.m. occurs between the second and the fourth day of incubation.

Distribution of the Virus in the Egg.—A group of 15 eggs that were incubated for 12 days prior to inoculation, were injected with a 10 per cent suspension of fascia infected with the sixth sheep alternating passage of the Egyptian sheep pox virus. They were incubated for four days at 36 C. From these eggs, chorioallantoic fluids, yolk sacs, embryos, and c.a.m. were obtained separately and, with the exception of the fluids, washed in three changes of saline, titrated, and 40 per cent suspensions were made from the yolk sacs and from the embryos and 20 per cent from the membranes. Ten per cent and tenfold dilutions were prepared from each suspension. The dilutions of each part were inoculated in 1 sheep (table 3).

The c.a.m. contained the highest amount of virus as the titer reached 10^{-4}, next came the yolk sac membrane which showed a titer of 10^{-2}, and the least amount of virus was found in the embryo and fluids as the titers reached only 10^{-1}.

The Effect of Age of Embryo on the Multiplication of Sheep Pox Virus.—Eighty eggs were allotted to four groups of 20 eggs each. The first group was incubated for eight days before inoculation, the second for ten days, the third for 12 days, and the fourth for 14 days. They were all inoculated on the c.a.m. with 10 per cent suspension of fascia infected with the sixth sheep alternating passage of Egyptian sheep pox virus. The eggs were incubated at 36 C. for four days, at the end of which time the membranes of each group were collected separately, triturated, and a 10 per cent suspension in saline was made. Tenfold dilutions were prepared from each group and titrated in a sheep (table 4).

The titer of virus in the four groups of membranes was the same; it reached 10^{-4}. These results indicate that at eight, ten, 12, or 14 days embryos could be used for the cultivation of the virus.

TABLE 4—The Effect of Age of Embryo on the Multiplication of the Sheep Pox Virus

Age of embryo (da.)	Sheep No.	Dilutions used	Results			Titer
			Area 1	Area 2	Area 3	
8	2591	1:10	+	+	+	10^{-4}
		1:100	+	+	+	
		1:1,000	+	+	+	
		1:10,000	+	+	+	
		1:100,000	—	—	—	
		1:1,000,000	—	—	—	
10	49	1:10	+	+	+	10^{-4}
		1:100	+	+	+	
		1:1,000	+	+	+	
		1:10,000	+	+	—	
		1:100,000	—	—	—	
		1:1,000,000	—	—	—	
12	1105	1:10	+	+	+	10^{-4}
		1:100	+	+	+	
		1:1,000	+	+	+	
		1:10,000	+	+	+	
		1:100,000	+	—	—	
		1:1,000,000	—	—	—	
14	1081	1:10	+	+	+	10^{-4}
		1:100	+	+	+	
		1:1,000	+	+	+	
		1:10,000	+	+	—	
		1:100,000	—	—	—	
		1:1,000,000	—	—	—	

+ = positive local reaction; — = negative local reaction.

The Effect of Temperature of Incubation After Inoculation of Embryos on the Development of Sheep Pox Virus.—Sixty fertile hens' eggs that were incubated for 12 days before inoculation were allotted to 3 groups of 20 eggs each. All the eggs were inoculated on the c.a.m. with a 10 per cent suspension of fascia infected with the sixth sheep alternating passage of Egyptian sheep pox virus.

The first group of eggs were incubated at 36 C., the second at 37 C., and the third at 39 C. They were all incubated for four days, at the end of which time the c.a.m. of each group were collected separately, triturated, and a 33.3 per cent suspension in saline was made. Ten per cent and then tenfold dilutions were prepared from each group of membranes and titrated in a sheep (table 5).

The virus multiplication in the eggs incubated at 36 C. or at 37 C. reached a high titer of 10^{-4}. Those membranes collected from the embryos incubated at 39 C. when inoculated in sheep 745 showed no skin or systemic reactions. This sheep when challenged with a known virus showed typical local and febrile reactions. These indicated clearly that at 39 C. incubation after

TABLE 5—The Effect of Temperature of Incubation After Inoculation of Embryo on the Development of Sheep Pox Virus

Temperature of incubation postinoculation	Sheep No.	Dilutions used	Results				
			Skin reaction				
			Area 1	Area 2	Area 3	Febrile reaction	Titer
36 C.	1145	33.3%	+	+	+	+	10^{-4}
		1:10	+	+	+		
		1:100	+	+	+		
		1:1,000	+	+	+		
		1:10,000	+	+	—		
		1:100,000	—	—	—		
		1:1,000,000	—	—	—		
37 C.	817	33.3%	+	+	+	+	10^{-4}
		1:10	+	+	+		
		1:100	+	+	+		
		1:1,000	+	+	+		
		1:10,000	+	+	+		
		1:100,000	—	—	—		
		1:1,000,000	—	—	—		
39 C.	745	33.3%	—	—	—	—	10^{-2}
		1:10	—	—	—		
		1:100	—	—	—		
		1:1,000	—	—	—		
		1:10,000	—	—	—		
		1:100,000	—	—	—		
		1:1,000,000	—	—	—		

+ = positive local or febrile reactions; — = negative local or febrile reactions.

inoculation there was no growth of virus on the c.a.m.

Cross-Immunity Tests.—In a previous report,[5] it was found that the Romanian strain of sheep pox virus cross immunized with the Egyptian strain. To find out whether the egg-cultured virus cross immunizes with the original Egyptian strain and the Romanian strain, the sheep which were used for the titration of the different egg passages and those which were used in the experiments on the study of the cultivation of the virus in eggs were allotted to five groups; two were challenged with 0.5 ml. of a 10 per cent suspension of the Egyptian sheep pox virus with a titer of 10^{-6}, and the other 2 groups were given similar doses of the Romanian sheep pox virus with a titer of 10^{-5}. Two control sheep were inoculated with similar doses, one with the

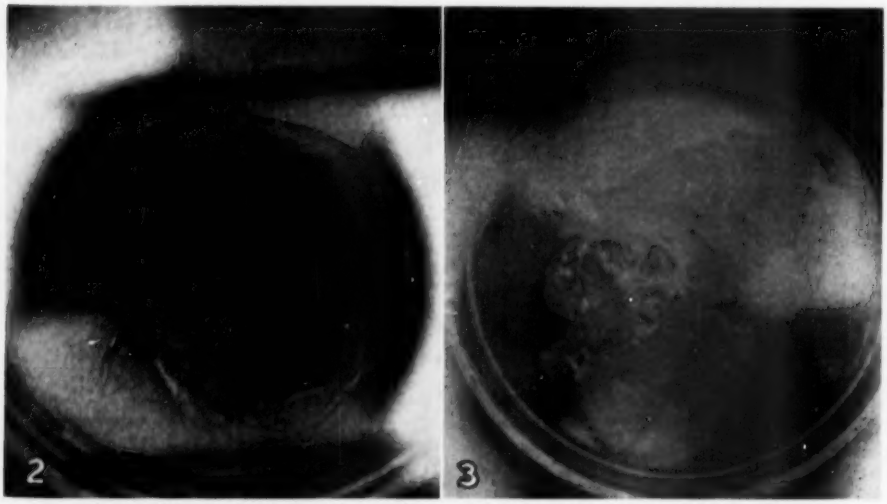


Fig. 2—Normal chorioallantoic membrane showing transparent and clear surface.
Fig. 3—Membrane inoculated with the Cairo S sheep pox virus, showing small and large pock lesions.

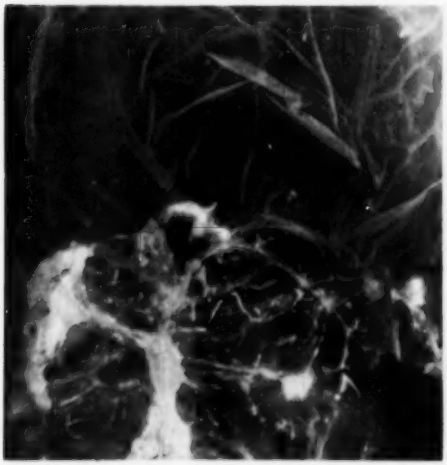


Fig. 4—Sheep pox-infected membrane with large confluent and small lesions. High power.

Egyptian virus and the other with the Romanian strain. None of the challenged sheep showed any reaction whether given the Egyptian virus or the Romanian strain. The 2 control sheep showed clearly positive local and systemic reactions. These results showed that the cultured sheep pox virus cross immunizes fully with both the original Egyptian and the Romanian strains.

The Susceptibility of Rabbits to Sheep Pox Virus.—The skin of the belly of rabbits (4 groups of 2 rabbits each) was shaved and scarified at several areas. Ten per cent suspensions of each of the original sheep pox virus, the egg virus representing the tenth c.a.m. alternating passage, the Romanian sheep pox virus, and the vaccinia virus were applied to the scarified areas of one group.

Only the 2 rabbits which were given the vaccinia virus showed skin and systemic reactions. None of the animals given the sheep pox virus showed any reaction. This shows clearly that rabbits are not susceptible to sheep pox virus even when cultured in eggs.

Trial of the Cultivation of the Romanian Strain of Sheep Pox Virus on the Chorioallantoic Membrane of Chicken Embryo.—Several attempts were made to cultivate the Romanian sheep pox strain of virus on the c.a.m. of chicken embryos using the same techniques and methods used in cultivating the Cairo S Egyptian strain, but all the results were negative, indicating that the Romanian strain does not grow in eggs.

Pathological Changes in the Chorioallantoic Membrane Produced by Sheep Pox Virus.—Four days after inoculation, the injected area of the membrane showed yellow, thickened, caseated patches, some of which were small like pock lesions, and others were confluent and occupied most of the area (fig. 3 and 4). No central necrosis was observed in these lesions. The virus had no ill-effect on the embryo as no mortalities were found among the inoculated embryos even after the sixth day of infection. Microscopically, the ectodermal layer,

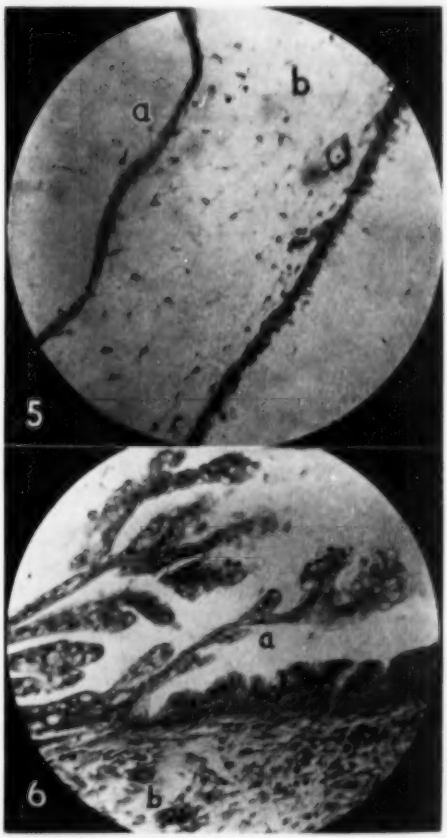


Fig. 5—Normal membrane showing one layer of flat cells in ectoderm (a) and few cells of fatty tissue in mesoderm (b).

Fig. 6—Sheep pox-infected membrane with proliferated ectodermal layer (a) and mesoderm (b) infiltrated with large numbers of leukocytes and fibroblasts.

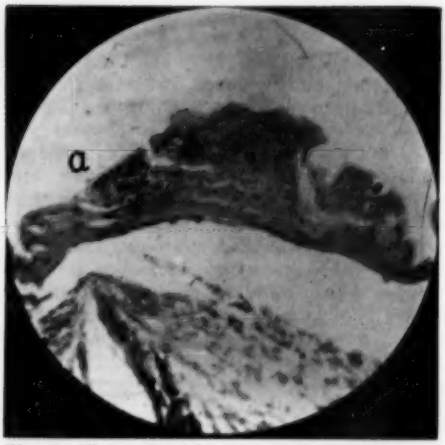


Fig. 7—Sheep pox-infected membrane with ectoderm (a) showing proliferation, degeneration, and necrosis.

which in the normal membrane is made of one layer of flat cells (fig. 5), showed in the sheep pox-infected membrane marked proliferation and was changed into several layers of cuboidal cells (fig. 6). When the membrane was examined four days after inoculation, the superficial cell layers were degenerated and necrosed (fig. 7). The mesoderm showed considerable hypertrophy and infiltration with leukocytes and fibroblasts. The blood vessels in the mesoderm were highly engorged and some of them showed ruptured walls. No marked changes were observed in the entodermal layer.

DISCUSSION AND SUMMARY

The Egyptian Cairo S strain of sheep pox virus was successfully cultivated on the chorioallantoic membrane (c.a.m.) of the developing chicken embryo for 21 passages, using the alternating method between sheep and chicken embryos. Until now, three direct passages of the virus in the membrane have been reached. The amount of virus obtained from the membrane was in some passages equal to that given by the sheep itself, as the titer reached 10^{-5} and 10^{-6}, and so, instead of the sheep used now, the embryo could be used as a cheap host for large-scale production of seed virus for vaccine preparation. No change in the character of the virus was observed by such egg passage, as the cultured virus still caused generalized infection in sheep and did not cause any reaction in rabbits. The cultured virus was found to cross immunize fully with both the original Egyptian virus and the Romanian strain.

In experimental studies of the cultivation of the sheep pox virus in the egg, it was found that two, three, or four days' incubation after inoculation was the best time for obtaining the maximum amount of virus from the membrane. Temperatures of 36 and 37 C. were found to be suitable for the incubation of the eggs after inoculation, whereas at 39 C., no virus grew on the membrane. The highest titer of virus was found in the c.a.m. and the least was found in the embryo and fluids. The virus produced characteristic lesions in the c.a.m. in the form of thickened, yellow, caseated patches of different sizes at the area of inoculation. Microscopically, the ectodermal layer showed proliferation, degeneration, and necrosis with considerable hypertrophy of the mesoderm. Several attempts were made to cultivate the Romanian strain of sheep pox virus on the c.a.m., using different methods with unsuccessful results.

References

[1] Hutyra, F., and Marek, J.: Variola Ovina. Special Path. and Therap. Dis. Domesticated Anim., *1*, (1938): 364.

[2] Ducloux, E., Cordier, G.: Sur le Virus Claveleux Traité par Diverses Aldéhydes. Compt. rend. Acad. sci., *183*, (1926): 486.

[3] Boto, J. T.: Un Nuevo Metodo de Immunizacion Activa Contra la Viruela Ovina. Trab. Inst. Biol. Anim., Madrid, *7*, (1942): 345–353.

[4] Donatien, A., Lestoquard, F., Plantureux, E., and Gayot, G.: Les Varioles Animales en Algérie. Arch. Inst. Pasteur d'Algérie, *25*, (1947): 62–70.

[5] Bacharan, M., Mavroglu, N., Inchildar, B., and Unel, S.: Vaccin Anticlaveleux Sensibilisé. Bull. Off. internat. Épizoot., *37*, (1952): 56.

[6] Delpy, L. P., and Rafyi, A.: Recherches Expérimentales sur le Virus Claveleux, Méthode d'Immunisation Employeé en Iran. Arch. Inst. Hessarek, *6*, (1949): 18–25.

[7] Delpy, L. P., and Mir Chamsy, H.: Stabilisation des Antigènes Claveleux et Préparation d'un Vaccin Mixte contre la Clavelée et la Fièvre Charboneuse. Arch. Inst. Hessarek, *6*, (1949): 15–17.

[8] Sabban, M. S.: Sheep Pox and Its Control in Egypt Using a Desiccated Live Virus Vaccine. Am. J. Vet. Res., *16*, (1955): 209.

[9] Rao, R. S.: Cultivation of Sheep Pox Virus on Chorioallantoic Membrane of the Chick Embryo. Ind. J. M. Res., *26*, (1938): 497.

[10] Blaxall, F. R.: A System of Bacteriology in Relation to Medicine, *7*, (1930): 140 (cited by Rao [9]).

[11] Findlay, G. M.: J. Roy. Microbiol. Soc., *56*, (1936): 213 (cited by Rao [9]).

[12] Gins, H. A., and Kunert, H.: Weitere Erfahrungen mit der Kuhpockenschutzimpfung bei Schafen. Deutsche tierärztl. Wchnschr., *45*, (1937): 257.

[13] Bennett, S. C. J., Horgan, E. S., and Mansur, A. H.: The Pox Diseases of Sheep and Goats. J. Comp. Path. and Therap., *54*, (1944): 131.

[14] Ortenzi, R., and Tibeco, G.: Tentativi di Coltivazione Del Virus Del Variolo Ovino Sulla M.C.A. Di Embrioni Di Pallo. Zooprofilassi, *9*, (1954): 669.

[15] Chamsy, H. M., Institut d'Hessarek, Iran: Personal communication.

[16] Nakamura, J., and Miyamoto, T.: Avianization of Lapinized Rinderpest Virus. Am. J. Vet. Res., *14*, (1953): 397.

Chlorinated Naphthalene Intoxication in Sheep

W. E. BROCK, D.V.M., M.S.; E. W. JONES, M.R.C.V.S., Ph.D.;
ROBERT MacVICAR, Ph.D.; L. S. POPE, Ph.D.

Stillwater, Oklahoma

NATURALLY OCCURRING cases of highly chlorinated naphthalene poisoning in sheep have not been observed.[1] This type of poisoning has been produced experimentally in sheep by feeding a processed wheat concentrate [2] which had also produced the condition in cattle. This study suggests that ten times more of the poisonous substance is required to produce toxicity in sheep than in cattle.

Polychlorinated naphthalene toxicity in cattle has been produced by a total dose as little as 0.45 mg. of chlorinated naphthalene per pound of body weight given during a seven-day period.[3] At this dosage, signs of intoxication appeared on the twelfth day after administration began. Based on the comparison between cattle and sheep and using the processed wheat concentrate, this would indicate that the toxic level in sheep was 5 to 10 mg. per pound of body weight.

Clinical signs in bovine chlorinated naphthalene intoxication include excessive lacrimation and salivation, nasal discharge, emaciation, proliferative lesions in mouth, diarrhea and, in the more chronic cases, the typical hyperkeratosis of the skin.[4] Gross lesions found at necropsy have included proliferative lesions in the esophageal mucosa, cirrhosis of liver, cystic swelling of mucosa of gallbladder and extra hepatic bile ducts, and striation in the renal cortex.[4-6] Histopathological findings have shown degeneration and cirrhosis of liver, proliferation of the intrahepatic bile ducts, cystic dilatation of mucosal glands of gallbladder and bile ducts, dilatation of the straight tubules of the kidney, squamous cell metaplasia of cervix and Gartner's ducts, and hyperkeratosis of the skin.[1,4-6]

Cattle poisoned with highly chlorinated naphthalene have consistently shown a decrease in vitamin A plasma levels to less than 10 μg./100 ml. of blood. This change occurs a few days after administration of the poison.[3]

Findings in the trial in which sheep have been poisoned experimentally included loss of appetite, straw-colored fluid in the body cavities, cirrhosis of the liver, and squamous cell metaplasia of the endometrium.[1,2]

Contamination of pelleted feed with certain types of grease containing the highly chlorinated naphthalenes during the pelleting process has been incriminated as a cause of bovine hyperkeratosis. This experiment was initiated to determine if a commercially pelleted feed would produce signs of bovine hyperkeratosis in sheep and to study the signs and lesions produced.

METHODS

Thirty ewes were equally allotted to six lots. They were weighed at that time and at approximately monthly intervals throughout the experiment.

The lots were assigned at random to separate indoor pens. Wheat straw was used as bedding. Cottonseed concentrate was fed, ¼ lb. per ewe per day for 39 days and then increased to ½ lb. per ewe per day for the remainder of the experiment. A poor quality alfalfa hay was fed at the rate of 3 lb. per ewe per day. At no time during the trial were the animals subjected to stress.

The concentrate feed was obtained from two plants of the Chickasha Cotton Oil Co. The 5 ewes in lot 1 were fed cottonseed pellets from the Anadarko plant; lot 2 ewes were fed cottonseed meal from the Chickasha plant; ewes in lots 3, 4, 5, and 6 were fed cottonseed pellets from the Chickasha plant.

Since 0.45 mg. of chlorinated naphthalene per pound of body weight was the smallest total dose used by Bell [3] to produce toxicity in cattle, 0.5 mg. per pound per day was chosen as the minimal dose for the sheep in this trial (lot 4). Ten times this amount (5.0 mg./lb./day) was given to the sheep in lot 5, and 25 times the smallest dosage (12.5 mg./lb./day) to those in lot 6. These dosage levels were maintained until death of the sheep terminated the experiment.

The chlorinated naphthalene (Halowax 1014*) was dissolved in corn oil to make a 10 per cent solution. The requisite dose of this solution was administered orally by gelatin capsule.

From the Oklahoma Agricultural Experiment Station, Stillwater.

The animals and feed used in this experiment were furnished by the Chickasha Cotton Oil Co.; the chlorinated naphthalene by the Halowax Products Division, Union Carbide and Carbon Corp.

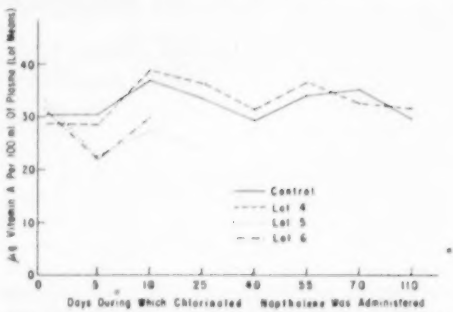
* Halowax Products Division, Union Carbide and Carbon Corp.

Each ewe was examined at weekly intervals for signs of disease, particularly lesions in the mouth, nostrils, and eyes that might be associated with vitamin A deficiency or hyperkeratosis as observed in cattle. Samples of blood for vitamin A analysis of the plasma were taken from each sheep before commencing the experiment and at five- to 15-day intervals during the experiment.

EXPERIMENTAL RESULTS

The results with the control sheep not given chlorinated naphthalene in lots 1, 2, and 3 have been reported in a previous paper.[7] There was no evidence of deleterious effects on the general health, weight, or plasma vitamin A levels. Two of the control sheep were killed for necropsy. No evidence of gross or microscopic pathological changes relative to this experiment was found in either sheep.

The amount of chlorinated naphthalene given to the sheep is summarized (table 1). Lot 4, given 0.5 mg. of the chemical per pound of body weight daily, required an average total amount of 5,108.0 mg. to produce death—an average total dose of 53.0 mg. per pound of body weight. Lot 5, given 5.0 mg. of chlorinated naphthalene per pound of body weight daily, received an average total dose of 13,596.0 mg., or 137.0 mg. per pound of body weight of the chemical, to result in death. Lot 6, given 12.5 mg. of chlorinated naphthalene per pound of body weight daily, required an average total dose of 25,878.0 mg., or 256.0 mg. per pound of body weight, to produce death.



Graph 1—The mean plasma vitamin A levels (μg./100 ml.) for each lot of sheep during the period of administration of chlorinated naphthalene.

The averages for lot 6 are based on 4 animals because sheep 26 died from foreign body pneumonia. Animal 16 was killed on the 135th day of treatment in order to terminate the trial. At the time of death, it was showing mild signs of toxicity.

The average number of days the sheep received chlorinated naphthalene before death was 106 for lot 4, 27 for lot 5, and 20 for lot 6.

Plasma Vitamin A.—The mean plasma vitamin A determinations of each lot made during this experiment are shown (graph

TABLE 1—Summary of the Amount of Chlorinated Naphthalene Administered Prior to Death to All Sheep in Treated Lots

Lot	Animal	Wt. (lb.)	Daily cl. naph./lb. (mg.)	Days treated	Total cl. naph. (mg.)	Total cl. naph./lb. (mg.)
4	16*	100	0.5	135	6,750	67
	17	85	0.5	113	4,802	56
	18	84	0.5	99	4,158	49
	19	123	0.5	94	5,781	47
	20	90	0.5	90	4,050	45
Mean	....	96.4	....	106	5,108	53
5	21	101	5.0	26	13,130	130
	22	132	5.0	23	15,180	115
	23	88	5.0	28	12,320	140
	24	87	5.0	35	15,225	175
	25	97	5.0	25	12,125	125
Mean	....	101.0	....	27	13,596	137
6	26†	79	12.5	7	6,912	87
	27	118	12.5	21	30,975	262
	28	95	12.5	25	29,687	312
	29	93	12.5	16	18,600	200
	30	97	12.5	20	24,250	250
Mean	....	100.75	....	20	25,878	256

* Killed to terminate the experiment. † Died of foreign body pneumonia.

1). There is no significant difference between the vitamin A values for the various lots on the day that chlorinated naphthalene feeding was started. Determinations of plasma vitamin A, made on the fifth and subsequent days of this experiment, showed it to be significantly lower in the higher dosage lots, 5 and 6, than in the control lots. There was no significant difference at any time between the plasma vitamin A determinations of the ewes in the control lots and those in lot 4 which received the low level of chlorinated naphthalene.

Although significant differences between plasma vitamin A levels of the ewes in control and treated lots were found, at no time was the plasma vitamin A at levels indicative of severe avitaminosis A. The lowest vitamin A level of 17.9 μg./100 ml. of plasma was found three days before death in 1 ewe.

Clinical Signs.—In all cases, the onset of toxic signs was manifested by a nasal discharge in excess of normal, weakness, loss of weight, and inappetence. The signs were more obvious and of longer duration in the lower intake groups.

Lot 6.—In the high level intake group, signs were observed only a short time before death. A serous nasal discharge, which was occasionally blood tinged, appeared and the animals became dyspneic. The mucous membranes were injected and icteric and the pulse weak and accelerated. Auscultation of the chest revealed increased vesicular sounds. The animals became recumbent and death occurred after eight to 24 hours of illness.

Lot 5.—The medium level intake group showed similar signs. The duration of illness was longer (2 to 3 days), and the weakness more obvious. Dyspnea, while still apparent, was less severe.

Lot 4.—In the low level intake group, dyspnea and icterus were not present. There was a loss of condition, with eventual recumbency and death after several days of illness. In some animals, abdominal distention was apparent and proved to be due to ascites.

Gross Pathology.—Gross lesions found at necropsy did not simulate those observed in cattle with chlorinated naphthalene poisoning. Lesions found in animals in all groups included liver damage, varying degrees of gastrointestinal hemorrhage, and increased pleural or peritoneal fluid or both.

Lot 6.—Throughout the gastrointestinal tract,[3] congestion and ecchymoses were common, especially in the abomasums and large intestines. The livers were consistently swollen, congested, and friable. They were mottled in appearance, dark areas of congestion and hemorrhage being intermingled with lighter cream-colored areas, suggestive of fatty degeneration. There were occasional ecchymoses in the fundi of the gallbladders and slight thickening of the bile ducts. An increased quantity of peritoneal fluid was present in 1 animal. The kidneys were congested and showed reddish brown streaks in the medulla. Uterine hemorrhage was observed in 1 ewe, while placental separation occurred in another. The larynx, trachea, and bronchi were congested and showed submucosal hemorrhages, and the lumens of the trachea and bronchi contained a frothy mucus in all animals. The lungs were congested and edematous, and straw-colored pleural fluid occurred in 3 ewes. In each animal, the heart was flaccid, the myocardium appeared degenerate, and numerous epicardial hemorrhages were present. The pericardial sacs contained an increased amount of fluid. The spleens were consistently congested and the blood did not clot. In 2 animals, degeneration and hemorrhage of the adrenal glands were noticed.

Lot 5.—The lesions involving the gastrointestinal tracts and livers were similar to the lesions in animals in the high intake group. The livers of 2 ewes appeared more firm and the consistency suggested an early cirrhosis. Sediment was occasionally seen in the gallbladders. There was an excessive amount of straw-colored peritoneal fluid with some coagulum in 2 animals. Congestion and degeneration of the kidneys were less obvious. Uterine hemorrhage occurred in only 1 animal. Lesions of the respiratory tracts and of the hearts were similar but were less severe. Congestion of the spleens was obvious in 2 animals. Hemorrhage and swelling of the adrenal glands occurred in 1 animal.

Lot 4.—Again, the lesions of the gastrointestinal tracts were similar but less severe than in either of the previous groups. The liver damage was again obvious, but congestion was less apparent and cirrhosis more obvious, the surface even assuming a slightly nodular appearance throughout

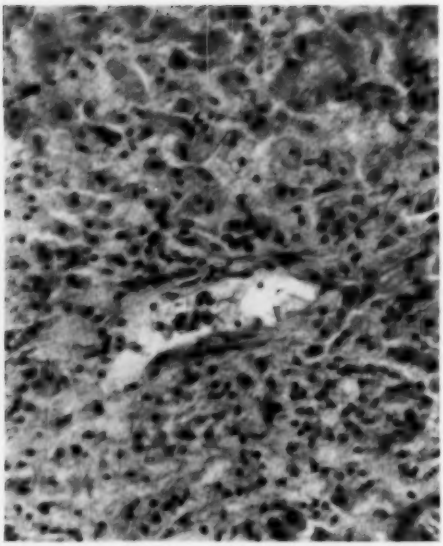


Fig. 1—Centrilobular fatty change, necrosis, and early fibrosis in the liver from sheep 23 which died 28 days after receiving 5.0 mg. of chlorinated naphthalene per pound of body weight daily. x 50.

the liver. Mottling still persisted. Occasionally, hemorrhage and thickening of the gallbladders and bile ducts was observed.

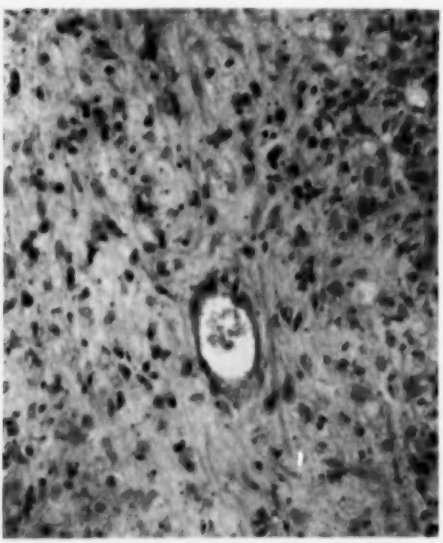


Fig. 2—Centrilobular necrosis and marked fibrosis in the liver from sheep 19 which died 94 days after receiving 0.5 mg. of chlorinated naphthalene per pound of body weight daily. x 50.

As distinct from the other groups, the mesenteries and omenta were thickened and contained petechiae. The peritoneal cavity in each animal contained large quantities of straw-colored fluid, some of which coagulated when the carcass was opened. No obvious renal lesions were observed, and hemorrhage into the uterine lumen was seen in only 1 animal. Congestion of the lungs was observed only once in this group, but increased pleural fluid occurred in 3 animals. There were no obvious lesions of either the hearts or spleens. The adrenal glands appeared degenerate in 1 animal.

Histopathology. — The histopathological changes were similar in all sheep receiving the toxicant, regardless of the dose administered. There was little difference between lots 5 and 6, as might be expected, since the sheep in these lots died within a relatively narrow range of time. The sheep in lot 4 showed much the same changes but in a more chronic form.

Lots 5 and 6.—There was marked congestion and hemorrhage in the livers of these sheep, with necrosis radiating from the central vein and all of the remaining hepatic cells showing some degree of degeneration (fig. 1, 2). A small amount of collagenous connective tissue was present around the central vein. In a few animals, there was congestion of the gallbladders and coagulation necrosis of the mucous membrane of the bile ducts.

The kidneys (fig. 3) showed marked congestion of the blood vessels, principally in the medulla below the arcuate artery. Heavy deposits of yellowish brown granules were found along the course of the congested blood vessels. These granules gave the cut kidney the characteristic brownish streaks (fig. 4) in the medullary pyramid found on gross examination.

Uteri from the sheep of these groups showed congestion of the endometrium with occasional hemorrhage into the lumen. The epithelium of many of the uterine glands was fragmented, with cells sloughed into the glandular lumen.

Marked congestion and bronchial exudate were consistently found in the lungs. Occasionally, there were areas of hemorrhage and of atelectasis. Two sheep had pneumonia.

Lesions common to the hearts of these sheep were cloudy swelling, hyperemia of the myocardium, and small hemorrhages

along the coronary vessels in the epicardia. Occasional small foci of myocardial necrosis were also found.

The spleens of these sheep invariably showed marked passive hyperemia, with occasional hemorrhage into the splenic substance. In most cases, there was atrophy of the malpighian corpuscles. Their adrenal glands showed hyperemia and various degrees of degeneration of the cortex.

Damage to their intestinal tracts was largely confined to the abomasums and colons where congestion, small hemorrhages, and degeneration of the mucosa were found.

Lot 4.—Much of the liver necrosis around the central veins, seen in the sheep of lots 5 and 6, was replaced in the sheep of lot 4 by collagenous connective tissue.

Less congestion was found in the renal medullas than in the sheep of lots 5 and 6, but damage to the proximal convoluted tubules and, to a lesser degree, the rest of the nephron was more pronounced.

Hemorrhage and congestion of the mucosa of the uteri were not found. Instead, there was sloughing of the glandular cells and deposition of a hyaline material into the lumen of the uterine glands.

Changes in the lungs, hearts, and spleens of the sheep were similar in all treated lots. In the spleens, however, there was an increase in the interstitial connective tissue and reaction centers. The adrenal glands were less hyperemic than those in sheep in lots 5 and 6 but there was an increased degeneration of the cortices.

DISCUSSION

The experiment primarily demonstrated that polychlorinated naphthalene poisoning in sheep is distinctly different from that in cattle. The amount of the chemical required to produce death in sheep was much greater and the pathological changes differed from those in cattle.

The smallest amount of chlorinated naphthalene per pound of body weight required to cause death in a sheep in this experiment was approximately 100 times greater than the smallest amount per pound of body weight reported by Bell to cause death of a calf. This difference between bovine and ovine lethal doses is much greater than that which might have been deduced from the results obtained with contaminated wheat concentrate by Olafson and McEntee.[2]

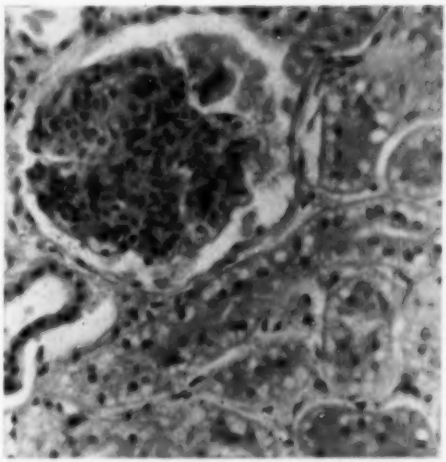


Fig. 3—Glomerulitis with protein in the lumen of Bowman's capsule and vacuolar change in the cells of the proximal convoluted tubules. This section is from the kidney of sheep 23 which died 28 days after receiving 5.0 mg. of chlorinated naphthalene per pound of body weight daily. x 50.

The range of the lethal dose for sheep within each lot shows a wide tolerance for the chemical among individuals. Considering the fact that about 4 Gm. of chlorin-

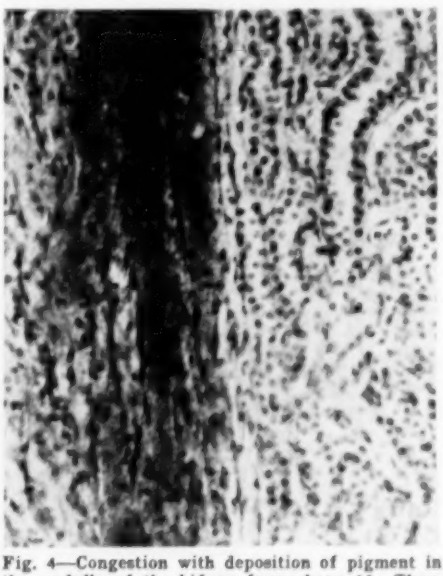


Fig. 4—Congestion with deposition of pigment in the medulla of the kidney from sheep 23. These pigmented striations appear as brown streaks in the medulla on gross examination. x 50.

ated naphthalene was the smallest lethal dose in these sheep, it is less likely that feed contamination, such as that which has been incriminated in bovine intoxication, would cause serious losses in sheep.

The two most characteristic pathological changes in bovine chlorinated naphthalene intoxication are cutaneous hyperkeratosis and extreme depression of plasma vitamin A levels. Hyperkeratosis was not observed in the sheep in this experiment. Plasma vitamin A levels, while significantly lower in the sheep given 5.0 mg. and 12.5 mg. of the chemical per pound of body weight than in the control sheep, did not show the extreme and rapid depression to values less than 10.0 μg./100 ml. of plasma characteristic of bovine intoxication. The sheep given 0.5 mg. of chlorinated naphthalene per pound of body weight did not show any significant depression in plasma vitamin A levels. Thus, the clinical changes most characteristic of this condition in cattle are either absent or comparatively insignificant in sheep.

The clinical and pathological changes were apparently due to toxic action of the chemical on the cardiovascular system, liver, and kidneys. Small hemorrhages found in the intestinal tracts, omenta, epicardial fat, uteri, kidneys, livers, and other organs indicate vascular damage. Lack of myocardial tone and cardiac dilatation found on necropsy, and microscopic indications of myocardial damage, illustrate the toxic effects on the heart. Toxic effects on the liver resulted in necrosis and cirrhosis around the central vein, while the kidneys showed degeneration of the nephrons. Cardiac insufficiency, coupled with increased resistance to portal blood flow through the liver, resulted in the general passive congestion of the spleens, kidneys, intestinal tracts, and lungs, and also in the large amounts of peritoneal and pleural fluids found at necropsy in the more chronic cases.

SUMMARY

Experimental intoxication in sheep from highly chlorinated naphthalene demonstrates that sheep have a much greater tolerance for the chemical than do cattle, and they do not show cutaneous hyperkeratosis or an excessive drop in the plasma vitamin A level. Affected sheep showed nasal discharge, weakness, loss of weight, inappetence, and ascites. The pathological findings were those associated with cardiovascular injury, necrosis and cirrhosis of the liver, and damage to the nephrons.

References

[1] McEntee, K., and Olafson, P.: Reproductive Tract Pathology in Hyperkeratosis of Cattle and Sheep. Fert. and Ster., *4*, (1953): 128–136.

[2] Olafson, P., and McEntee, L.: The Experimental Production of Hyperkeratosis (X-Disease) by Feeding a Processed Concentrate. Cornell Vet., *41*, (1951): 107–109.

[3] Bell, W. B.: Further Studies on the Production of Bovine Hyperkeratosis by the Administration of a Lubricant. Virginia J. Sci., *3*, (1952): 169–177.

[4] Olafson, P.: Hyperkeratosis (X-Disease) of Cattle. Cornell Vet., *37*, (1947): 279–291.

[5] Morrill, C. C., and Link, R. F.: X-Disease (Hyperkeratosis of Cattle in Illinois. Univ. of Illinois Circ. 656, 1952.

[6] Olson, C., Jr., and Cook, R. H.: Attempts to Produce Bovine Hyperkeratosis. Am. J. Vet. Res., *12*, (1951): 261–272.

[7] Brock, W. E.: A Preliminary Report on Studies on Chlorinated Naphthalene Toxicity in Sheep. Oklahoma Agric. Exper. Sta. mimeo. circ. M-271, 1955.

Leukocytic Response in Lambs Exposed to Whole-Body X Irradiation

L. A. GEORGE, Jr., D.V.M.; PATRICIA L. HACKETT, M.S.; L. K. BUSTAD, D.V.M.

Richland, Washington

IN PREVIOUS STUDIES[1] conducted at this laboratory, lambs 8 to 10 weeks old were exposed to a multiple radiation assault which included exposure to 10, 50, or 100 r of whole-body x irradiation, *beta* irradiation of skin, and oral ingestion of I^{131}. One of the principal purposes of the studies was the demonstration of the pattern of initial leukocytic response after exposure to a sublethal dose of whole-body x irradiation. The total radiation dose derived from the *beta* exposure of skin and the ingestion of I^{131} was not large enough to have affected the leukocytic response of the animals[2]; thus, the results observed were attributed to the whole-body, x-ray exposure.

In the initial studies, an appreciable quantitative difference in the early leukocytic response of the animals following exposure to the different whole-body dose levels was demonstrated. Compared with preirradiation levels, the response ranged from no significant depression at the 10 r level to about 50 per cent depression at the 50 r level and a 60 to 70 per cent depression at the 100 r level.

These studies stimulated interest relative to the dose level at which the first perceptible leukocytic depression would occur. The study reported in this paper represents more recent work on this problem and presents the results obtained by exposing young male lambs to bilateral, whole-body, x-ray exposures of 15 and 30 r.

TABLE 1—Number of Animals and X-Ray Exposure per Group

Group	No. of animals	X-ray dose
1	6	None
2	6	15 r
3	6	30 r

The conditions of exposure were: Maxitron settings: 250 kv., 30 ma.; filtration: 2.5 mm. Cu and 1.0 mm. Al; distance to midline of animal: 183 cm.; dose rate: 3.8 r/minute (in air); half-value layer: 3.5 mm. Cu; and effective energy (calculated in air): 145 kev.

MATERIAL AND METHODS

A total of 18 young, male, nursing Suffolk lambs, weighing between 40 and 55 kg. each, comprised the experimental population. The dis-

From the Biology Operation, Hanford Laboratories, General Electric Co., Richland, Wash.

Based on work performed under contract W-31-109-Eng-52 for the Atomic Energy Commission.

The technical assistance of M. E. Kerr and the Animal Farm staff and of the Clinical Laboratory personnel is gratefully acknowledged by the authors.

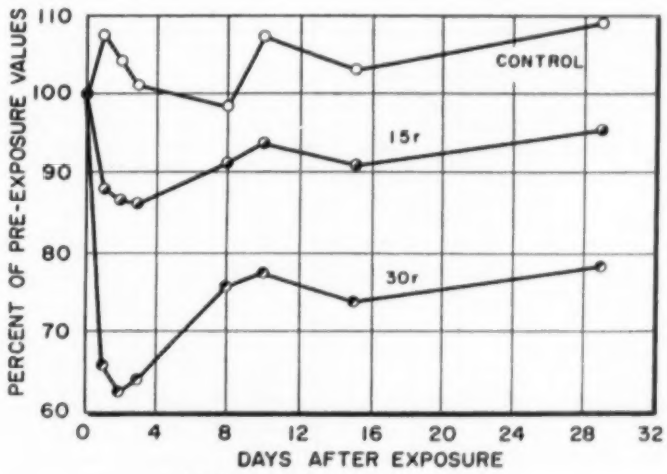


Fig. 1—Percentage of pre-exposure lymphocyte values in control and exposed groups.

tribution of the animals and other pertinent features of the experimental design are shown (table 1). The animals were immobilized during the x-ray exposure period by being placed in a canvas sling which was suspended in front of the x-ray generator (General Electric-Maxitron). The total dose was divided equally between the right and left sides of the animals. Control animals were similarly immobilized and suspended in the x-ray generator facility but were not irradiated.

Three blood samples were obtained from each of the 18 animals during the week prior to exposure to obtain the pretreatment data. The animals were then grouped by random selection. All animals were examined daily throughout the post-irradiation phase of the experiment and blood samples were taken, at established intervals, by jugular puncture, using a 21-gauge needle and evacuated tubes. Heparin was used as an anticoagulant. The leukocytes were diluted with a solution of 0.05 per cent methyl violet in 5.0 per cent acetic acid for enumeration. For the determination of packed volume of erythrocytes, Wintrobe's hematocrit tubes were centrifuged at 2,700 r.p.m. for 30 minutes. Hemoglobin concentrations were determined by the method recom-

TABLE 2—Hematological Values

Constituent	Days After Exposure	No. of Samples	Control	15 r	30 r
Packed-cell volume	pre-treatment	18	37.4 ± 1.3*	40.1 ± 1.9*	40.2 ± 1.7*
	1	6	39.3 ± 1.4	42.8 ± 4.3	40.4 ± 3.2
	2	6	35.8 ± 2.6	38.0 ± 4.3	37.6 ± 3.0
	3	6	35.5 ± 3.5	36.5 ± 3.6	36.7 ± 4.4
	8	6	35.0 ± 3.8	36.3 ± 3.5	36.7 ± 3.5
	10	6	35.7 ± 3.5	38.9 ± 3.3	37.4 ± 2.5
	15	6	34.2 ± 2.1	35.6 ± 3.5	35.5 ± 2.7
	29	6	41.1 ± 6.0	41.5 ± 4.7	44.4 ± 4.6
Hemoglobin (g/100 ml)	pre-treatment	18	12.05 ± 0.20	12.62 ± 0.41	12.32 ± 0.44
	1	6	12.45 ± 0.45	13.18 ± 0.96	12.82 ± 0.66
	2	6	11.80 ± 0.63	12.41 ± 1.01	12.22 ± 0.69
	3	6	11.95 ± 0.48	12.05 ± 1.15	12.12 ± 1.05
	8	6	11.69 ± 0.79	12.12 ± 1.07	11.67 ± 0.66
	10	6	11.99 ± 0.90	13.01 ± 0.69	12.31 ± 0.73
	15	6	11.36 ± 0.54	11.72 ± 0.71	11.65 ± 0.73
	29	6	11.00 ± 0.48	11.46 ± 0.79	11.53 ± 1.03
Leukocytes (cells x 10^{-3}/mm^3)	pre-treatment	18	9.58 ± 0.81	11.35 ± 0.81	12.76 ± 1.04
	1	6	10.08 ± 1.46	10.20 ± 2.20	9.51 ± 2.12
	2	6	9.67 ± 1.49	9.55 ± 2.27	8.97 ± 1.90
	3	6	9.53 ± 1.27	9.82 ± 1.67	9.14 ± 1.58
	8	6	9.03 ± 0.94	10.20 ± 2.12	10.08 ± 2.49
	10	6	9.44 ± 1.64	10.07 ± 2.32	10.44 ± 2.59
	15	6	9.95 ± 1.53	9.99 ± 2.27	10.82 ± 1.90
	29	6	10.02 ± 1.07	9.68 ± 1.22	9.99 ± 0.86
Neutrophils (cells x 10^{-3}/mm^3)	pre-treatment	18	2.80 ± 0.29	3.98 ± 0.36	3.18 ± 0.51
	1	6	2.78 ± 0.43	3.13 ± 0.89	3.24 ± 0.99
	2	6	2.66 ± 0.74	2.63 ± 0.97	2.97 ± 1.18
	3	6	2.74 ± 0.43	2.85 ± 0.65	3.01 ± 0.81
	8	6	2.38 ± 0.35	2.89 ± 0.71	2.81 ± 1.25
	10	6	2.19 ± 0.40	2.57 ± 0.43	2.94 ± 1.05
	15	6	2.97 ± 1.26	2.73 ± 0.74	3.81 ± 1.78
	29	6	2.60 ± 0.29	2.70 ± 0.44	2.52 ± 1.09
Lymphocytes (cells x 10^{-3}/mm^3)	pre-treatment	18	6.61 ± 0.60	7.81 ± 0.60	9.33 ± 0.88
	1	6	7.11 ± 1.55	6.87 ± 1.47	6.15 ± 1.11
	2	6	6.89 ± 1.04	6.76 ± 1.63	5.85 ± 0.61
	3	6	6.67 ± 1.18	6.73 ± 1.91	5.98 ± 1.01
	8	6	6.50 ± 0.79	7.12 ± 2.04	7.07 ± 1.59
	10	6	7.11 ± 1.51	7.31 ± 1.99	7.32 ± 1.58
	15	6	6.82 ± 1.16	7.10 ± 1.84	6.87 ± 1.44
	29	6	7.24 ± 1.13	7.47 ± 1.27	7.34 ± 0.77

* 95% confidence intervals about the mean

Correction: In table 2 above, the first entry for neutrophils under the 15 r heading should be 2.98 instead of 3.98.

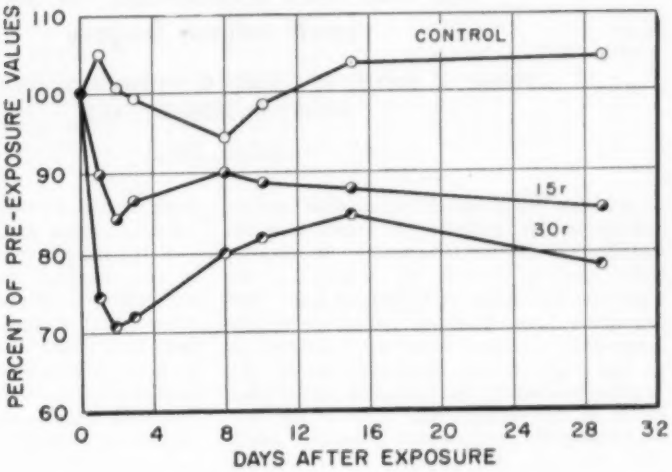


Fig. 2 — Percentage of pre-exposure leukocyte values in control and exposed groups.

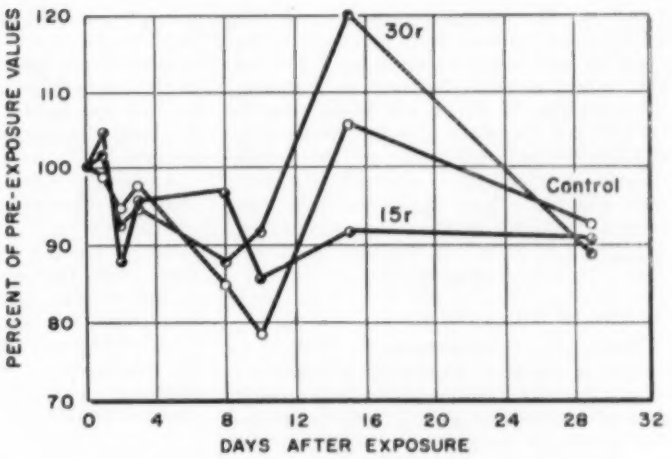


Fig. 3 — Percentage of pre-exposure neutrophil values in control and exposed groups.

mended by Cannan.[2] Student's t-test[4] was used to evaluate the significance of differences between mean values. Differences referred to as significant had P equal to or less than 0.01.

RESULTS AND DISCUSSION

No clinical manifestations attributable to the whole-body exposure were observed during the postexposure period. The animals remained alert, maintained their appetite, made normal weight gains, and appeared contented.

The lambs which received 30 r of total body x irradiation showed a significant decrease in lymphocytes when the three postirradiation values were compared with the pretreatment values (fig. 1). This decrease is reflected in total leukocyte levels (fig. 2). The decrease in lymphocytes in the animals that received 15 r is not significant with the numbers of animals utilized.

No significant changes occurred in neutrophil (fig. 3), packed cell volume, and hemoglobin levels (table 2).

References

[1] George, L. A., Jr., Hackett, P. L., and Bustad, L. K.: Triple Radiation Assault on Debilitated Lambs. U. S. Atomic Energy Comm. Document HW-42540, 1955 (unclassified).

[2] George, L. A., Jr., Hackett, P. L., and Bustad, L. K.: Leukocytic Response Following Multiple Radiation Assault. To be published.

[3] Cannan, R. K.: Proposal for the Distribution of a Certified Standard for Use in Hemoglobinometry. J. Lab. and Clin. Med., *46*. (1955): 135.

[4] Fisher, R. A.: Statistical Methods for Research Workers. 9th ed. Oliver, Edinburgh and London, 1944.

An Electronic Stethoscope for Sterile Small Animal Surgery

JIMMY A. DAVIS; BENJAMIN D. FREMMING, D.V.M., M.P.H.; HENRY M. BORELLA, M.S.

Austin, Texas

A METHOD of monitoring cardiac action during sterile surgery on small animals was desired at this laboratory. It was decided that a method of displaying the heartbeat by means of a flashing light during sterile surgery would suffice. Adequate diagnostic facilities were not available to us for auscultation of the chests of the smaller laboratory animals at a cost within reach of our budget, and none of the several electronic stethoscopes currently available were thought capable of adequately fulfilling our requirements.

Animal size dictated that a small, flat microphone, which could be taped to the chest of an animal as small as a 250-Gm. rat, would have to be used as a pickup. The impulse of the heart could then be transferred by means of a cord to an amplifying system, well clear of the sterile-draped surgical field, activating a neon bulb and radio-type earphones. With these criteria in mind, an electronic stethoscope, which will be described, was developed with cost a foremost consideration.

NATURE OF HEART SOUND

The sound emitting from the chest of an animal is complex. Preliminary studies indicate that the fundamentals of the heartbeats of animals studied lie between 30 and 250 cycles per second (cps.) with harmonic extending upward to several hundred cycles. Moreover, râles and other thoracic noises complicate the sound in a frequency range from 200 to 1,000 cps. Hence, this sound is an admixture of many sounds.

While the ear can be trained to interpret sound, an electronic device can not distinguish a sound purely by its source. In order to obtain a visible indication of the heartbeat, unique properties of its wave form must be utilized to distinguish it from the other noises which accompany it.

From the above, it can be seen that most of the acoustic energy from the heart is expended in frequencies below that of the other chest sounds. Moreover, the heartbeat is a short, intense sound, which can be heard above the other noises. These two characteristics of the heart sound were selected to actuate the mechanism for isolating the heartbeat.

Before the heartbeat can be heard or "flashed," it must first be detected. The over-all required elements of an electronic stethoscope are: detector (microphone), amplifier, discriminator circuits for isolating the heartbeat, and a triggering circuit to flash the light. Moreover, a phone outlet from the amplifier is needed for aural output.

It can be readily seen from the above that the requirements for the microphone or detector are critical. Good, low frequency response down to 30 cps. is necessary, and the unit should be pressure actuated. The microphone used in this particular instrument is the Maico No. 55-16 which is manufactured especially for cardiophony. Tests show that this microphone performs especially well with small animals.

CIRCUITRY

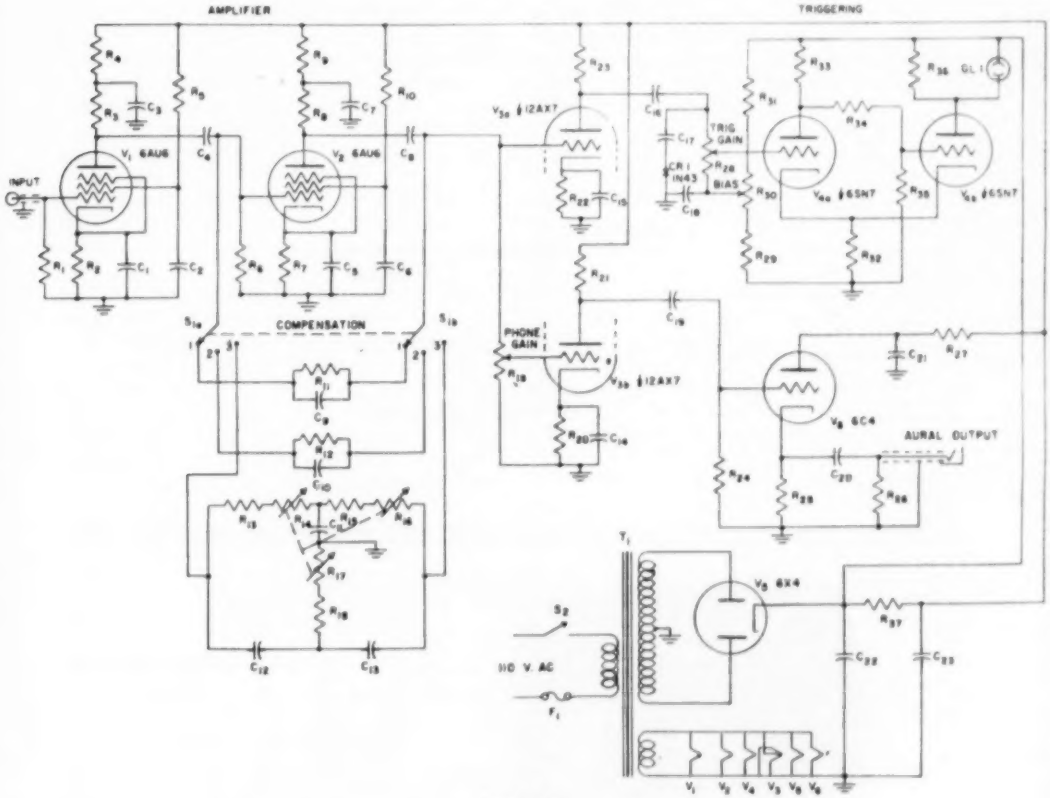
No unique circuitry is used in this instrument; however, various simple stages were selected for their particular suitability. Basically, v_1 and v_2 (*see* fig. 1) make up a high-gain amplifier which serves as the input and also provides frequency compensation. The signal then goes into both an input stage for the light output and an input stage for the aural output.

Frequency compensation is accomplished by a single feedback loop around v_2. Three RC networks can be switched (s_1) into this loop to give the response curves shown in figure 2. Position 1, with a band width

From the Radiobiological Laboratory of the University of Texas and the U. S. Air Force, Austin, Texas (Davis and Borella), and South Hills Animal Hospital, Pittsburgh, Pa. (Fremming).

This study was supported (in part) by funds provided under contract AF 18(600)-165 with the U.S.A.F. School of Aviation Medicine, Randolph Air Force Base, Texas.

from 20 cps. to 1,500 cps., is designed to allow most of the thoracic noise to be heard. It can be used for diagnostic work if desired. Position 2 limits the band width to the lower frequencies, allowing little sound other than the heartbeat to pass. Position 3 converts v_2 into a narrow band-pass filter which allows only about one octave band width, its center band frequency being adjustable, with ganged potentiometers R_{14}, R_{16}, and R_{17}, from 35 to 250 cps. The purpose of this position is to allow the fundamental of the heartbeat to be "tuned" to the circuit and to



Parts List

C_1, C_5, 40-40 mfd., 25 v. electrolytic.
C_2, C_6, C_{18}, C_{20}, .25 mfd., paper.
C_3, C_7, C_{21}, 10-10-10 mfd., 450 v. electrolytic.
C_4, C_8, C_{11}, C_{19}, .1 mfd., paper.
C_9, .00013 mfd., ceramic disc.
C_{10}, .001 mfd., ceramic disc.
C_{12}, C_{13}, .05 mfd., paper.
C_{14}, C_{15}, 20-20 mfd., 150 v. electrolytic.
C_{16}, .05 mfd. paper.
C_{17}, .015 mfd., paper.
C_{22}, C_{23}, 40 mfd., 450 v. electrolytic.
R_1, 330,000 ohm, 1/2 w.
R_2, R_7, 4,700 ohm, 1/2 w.
R_3, R_8, R_{24}, 220,000 ohm, 1/2 w.
R_4, R_9, R_{21}, R_{22}, 150,000 ohm, 1/2 w.
R_5, R_{10}, 470,000 ohm, 1/2 w.
R_6, R_{34}, R_{36}, R_{31}, R_{35}, 100,000 ohm, 1/2 w.
R_{11}, R_{12}, 680,000 ohm, 1/2 w.
R_{13}, R_{15}, 10,000 ohm, 1%, 1/2 w.
R_{14}, R_{16}, 100,000 ohm potentiometers, linear taper.
R_{17}, 50,000 ohm potentiometers, linear taper (R_{14}, R_{16}, R_{17} ganged).
R_{18}, 5,000 ohm, 1%, 1/2 w.
R_{19}, R_{29}, 100,000 ohm potentiometers, audio taper.
R_{20}, R_{23}, 1,500 ohm, 1/2 w.
R_{25}, 10,000 ohm, 1 w.
R_{27}, 82,000 ohm, 1 w.
R_{28}, 250,000 ohm, 1/2 w.
R_{30}, 1 megohm potentiometer, linear taper.
R_{32}, 5,000 ohm, 1 w.
R_{33}, 33,000 ohm, 1 w.
R_{38}, 22,000 ohm, 1 w.
R_{37}, 20,000 ohms, wire wound, 15 w.
V_1, 6AU6 vacuum tube.
V_2, 6AU6 vacuum tube.
V_3, 12AX7 vacuum tube.
V_4, 6SN7 vacuum tube.
V_5, 6X4 vacuum tube.
V_6, 6C4 vacuum tube.
GL1 NE34 neon tube.
T_1, power transformer, 500 v. ct. at 40 ma., 6.3 v. at 3 amp.
CR_1, 1N43.
S_1, 2-pole triple throw switch.
F_1, 1/2 amp. fuse.

Fig. 1—Circuit diagram.

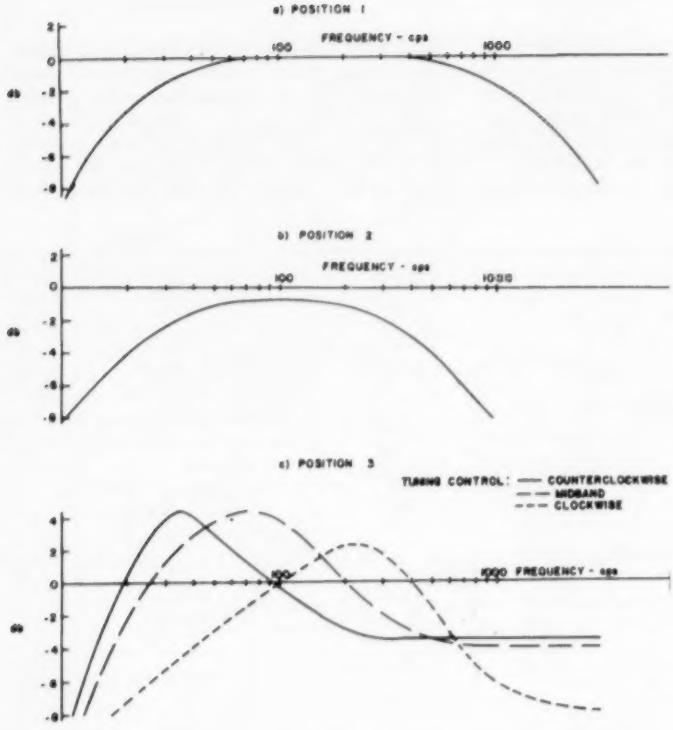


Fig. 2—Relative gain versus frequency for different compensation positions.

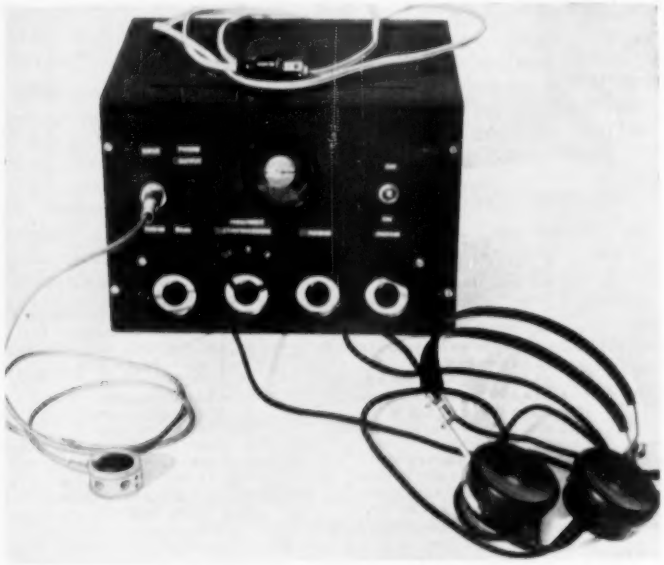


Fig. 3—Electronic stethoscope.

receive far more amplification than other frequencies.

Tube V_{2a} serves as an amplifier input to the triggering circuit. The triggering circuit itself is a simple Schmitt triggering circuit of the pulse-height discriminator type. The positive bias on V_{4a} is such that only negative signals with amplitudes equal to or greater than this bias will pass. This bias is adjustable; therefore, since the heartbeat is the most intense sound emitted from the chest and has been further isolated by frequency compensation, it can be isolated completely. Only when the heart beats will V_{4b} conduct (flashing the neon bulb).

In most animals, the systolic beat is more intense than the diastolic, and it alone will flash the light. However, in some animals, the light will flash on both beats, yet each heartbeat cycle can be readily distinguished up to 500 beats per minute.

In addition to the bias control for the triggering circuit, a gain control (R_{28}) mounted on the rear of the chassis adjusts the amplitude of the over-all incoming signal. This is primarily for adjusting the signal height from species to species, leaving the bias control for fine adjustments.

The phone output consists of one stage of amplification and a cathode follower designed to feed into medium-impedance headphones. A separate gain control for the phones is provided by R_{19}.

TECHNIQUE OF EMPLOYMENT

A small area of the chest wall is clipped of hair with a No. 40 Oster clipper, and the microphone described above is attached either with an elastic band or with masking tape to the thorax. The cord relaying the impulses from the microphone to the amplifying system is extended from the operating table to a convenient location that is within sight of the surgeons performing the operation. The amplifier is adjusted and, in some cases, if the systolic frequency is different from the diastolic, one or the other may be masked out, as indicated later, so that a "true" pulse rate is observed visually on the flashing neon bulb.

For purposes of auscultation, the headphone attachment is utilized, and auscultation is accomplished as is normally done with a Ford-type stethoscope. We have not found it mandatory to shave short-haired animals such as the monkey, rat, guinea pig, hamster, and domestic cat; but in the long-haired dogs and rabbits, we have felt that clipping or shaving considerably improves the transfer of the heart impulses to the amplifying system.

Compensation positions 1 and 2 are designed for phone use, while position 2, serving a dual purpose, and position 3 are employed for best actuation of the light.

The instrument should be warmed up for a few minutes prior to using. After warming up, with compensation switch in position 1, the heart is monitored with the microphone at various locations on the chest until the point of maximum heartbeat is found. The microphone is attached at this point. The compensation is then changed to position 2 or 3 depending on purpose. If it is desired to listen to the heart intermittently while using the indicating light, position 2 is preferred; if no ausculation is planned, then position 3 is suggested. If position 3 is used, the tuning control (R_{14}, R_{16}, and R_{17} ganged) must be adjusted for maximum signal from the heart. With the compensation switch in position 2 or 3, the bias is adjusted until only one light flash is seen (or in some cases, 2, as explained above) for each systolic heartbeat heard on the phones. If no flash can be obtained, an increase of the trigger gain is indicated. If the light can not be limited to flashing with the systolic heartbeat, lowering of the gain is indicated.

This stethoscope has been utilized in a number of surgical procedures on the dog, cat, hamster, guinea pig, rabbit, rat, and monkey and has been found unsatisfactory only in thoracic surgery. The large arteries and the heart itself are usually visible during thoracic surgery on smaller animals, and the need for monitoring the heartbeat, fortunately, is reduced.

SUMMARY AND CONCLUSIONS

An electronic stethoscope for use in sterile surgery of the rabbit, rat, guinea pig, hamster, dog, cat, and monkey has been described. The instrument as described is of minimal cost and can be assembled by most hobbyists for a cost not exceeding $60.

We have found this instrument to be of value in our sterile surgery work with small animals at this laboratory.

Determination of Maltase Activity in the Blood Serum of the Dog

RALPH G. SKILLEN, V.M.D., M.S.

Pasadena, California

MALTASE is an α-D-glucosidase which catalyzes the hydrolysis of 1 mole of maltose to 2 moles of glucose. Its presence in the blood serum of the dog was first demonstrated by Compton [1] in 1921. This enzyme has also been shown to be present in the serums of the pig, rat, mouse, horse, ox, sheep, and goat. It is present in only small amounts in the serum of the cat and is not found in the serums of man, monkey, rabbit, guinea pig, chicken, and pigeon.[1-4]

Although the natural occurrence of this enzyme in the serum has been studied, little information has been obtained relative to its biochemical properties or its physiological significance. In order to acquire such information, it was necessary to develop a convenient standard method whereby the activity of this enzyme could be determined. The purpose of this report is to present such a method for the measurement of serum maltase activity and to present information concerning the effects of pH and substrate concentration on the activity of this enzyme.

MATERIALS AND METHODS

The method employed to determine the maltase activity of a sample of dog serum depends on the increase in the reducing power of a mixture containing dog serum and the substrate, maltose, after incubation.

Dog serum was collected and prepared for use while taking all available precautions to insure the sterility of the serum sample. After reserving a portion of the serum with which to determine the concentration of the reducing substances present, the remaining portion was diluted in the ratio of 1 part of serum to 99 parts of saline solution (0.9% NaCl). This diluted sample was either used immediately or frozen and stored for future use; no loss of activity was observed in the frozen samples.

The concentration of the reducing substances in the serum was determined, using the fresh undiluted sample. A protein-free filtrate was obtained by the method of Somogyi,[5] and the concentration of the reducing substances was otherwise determined by the colorimetric adaptation of the Somogyi method for the determination of glucose described by Nelson.[6] A Klett photoelectric colorimeter equipped with a No. 54 green filter was used in the determination of the reducing substances.

To prepare the test incubation mixture, a diluted serum sample was adjusted to the desired pH with a 0.2 M phosphate buffer (Na_2HPO_4/KH_2PO_4), the final solution being 1 part of diluted serum to 5 parts of this buffer. After this solution had been warmed to 39 C. in a constant-temperature water bath, 2 volumes of a maltose solution of the desired concentration, freshly prepared and similarly warmed, were added and mixed. For every test incubation mixture of a given maltose concentration, a simultaneous control incubation mixture was utilized. The control incubation mixtures were prepared so that the maltose concentrations were the same as the initial maltose concentrations of the corresponding test mixtures. However, instead of containing the diluted serum, the control mixtures contained saline without serum.

The test and control mixtures were incubated simultaneously at 39 C. At the appropriate time intervals, 8.0 ml. were withdrawn from the incubation mixtures and added to 1.0 ml. of a 10 per cent $ZnSO_4 \cdot 7H_2O$ in order to precipitate the proteins and stop the reaction. After mixing, 1.0 ml. of 0.5 N NaOH was added and mixed; the solution was then filtered and the protein-free filtrate obtained. Simultaneous sampling of the test and control mixtures served to insure against possible error resulting from the hydrolysis of maltose by microbial action.

The protein-free filtrates of both the test and control mixtures were then used to determine the concentration of the reducing substances in the incubation mixtures; the same procedure was followed as with the serum protein-free filtrate. When the higher concentrations of maltose were used in the test and control mixtures, it was necessary to dilute the protein-free filtrate in the ratio of 1 part of filtrate to 4 parts of water. The colorimeter readings obtained from such samples were multiplied by five.

In all experiments, duplicate determinations were made on each sample.

Calculation of the Molar Concentration of Reducing Substances in Serum.—Using glucose solutions of known concentrations and treating them in the same manner as serum, a standard curve

From the Institute of Medical Research of the Collis P. and Howard Huntington Memorial Hospital, Pasadena, Calif.

This work was supported by funds from the A. H. Robins Co. of Richmond, Va.

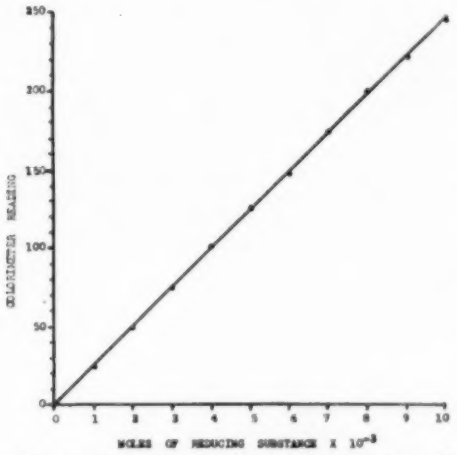


Fig. 1—Standard curve for the determination of the concentration of reducing substances (glucose) in 1 ml. of solution.

(fig. 1) was constructed. With the instruments used, it was found that under these circumstances the molar concentration of glucose in a solution was equal to the colorimeter reading multiplied by 4.05×10^{-5}.

Calculation of the Molar Concentration of Reducing Substances in Test and Control Samples.—Inasmuch as 8.0 ml. were used in the sampling of the test and control incubation mixtures, the concentrations of the reducing substances in these mixtures were 0.125 of the concentrations of serums with the same colorimeter reading; *i.e.*, the molar concentration of the reducing substances in the test and control mixtures was equal to the colorimeter reading multiplied by 5.06×10^{-6}.

Calculation of the Moles of Reducing Substances Present in the Test and Control Samples.—The number of moles of reducing substances present in the test and control samples was equal to the molar concentration of reducing substances in these samples multiplied by 8×10^{-4} or the colorimeter reading multiplied by 4.05×10^{-5}.

Calculation of the Moles of Maltose Hydrolyzed.—The number of moles of maltose hydrolyzed may be calculated by the equation:

$$M_h = M_f - (M_s + M_i).$$

M_h represents the number of moles of maltose hydrolyzed; M_f, the final number of moles of reducing substance after hydrolysis; M_s, the number of moles of reducing substance contributed by the serum; and M_i, the initial number of moles of maltose as determined from the control value.

Since only 0.01 ml. of serum is actually used in the test mixture, M_s equals the molar concentration of the reducing substances in the serum $\times 10^{-6}$.

Calculation of the Moles of Glucose Produced.—The moles of glucose produced is equal to $2M_h$.

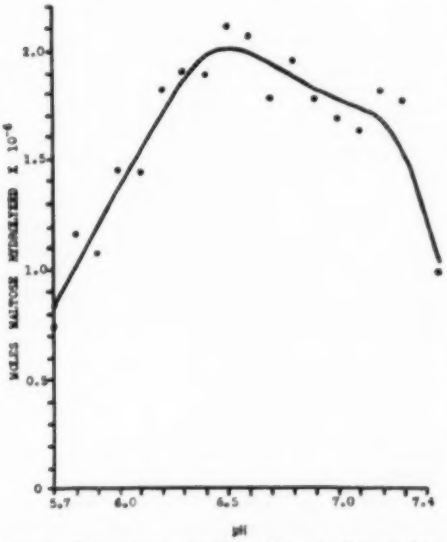


Fig. 2—The effect of pH on the activity of dog serum maltase. Maltose concentration = 6.53×10^{-4} M; incubation temperature = 39 C.; and period of incubation = 60 minutes.

RESULTS

The Effect of pH on Maltase Activity.—Three experiments were performed to determine at what pH the maltase activity was maximal. The pH of the incubation mixture was varied from 5.7 to 7.4, determinations being made at each 0.1 pH unit. Each experiment utilized a serum sample from a different dog; the substrate concentration was 6.35×10^{-4}. The curve showing the effect of pH on maltase activity (fig. 2) was constructed using the average values of the three experiments.

Throughout the entire pH range studied, there existed good maltase activity. The optimal pH appeared to lie between pH 6.4 and 6.6; however, strong activity was observed between pH 6.1 and 7.3. These findings are in agreement with those of Kokuryo[7] who similarly observed strong activity through a wide pH range with a maximal activity at approximately pH 6.6.

The Effect of Substrate Concentration on Maltase Activity.—Of extreme importance in the determination of dog serum maltase activity is the concentration of the substrate. The results (fig. 3) show that although maltose is usually hydrolyzed by

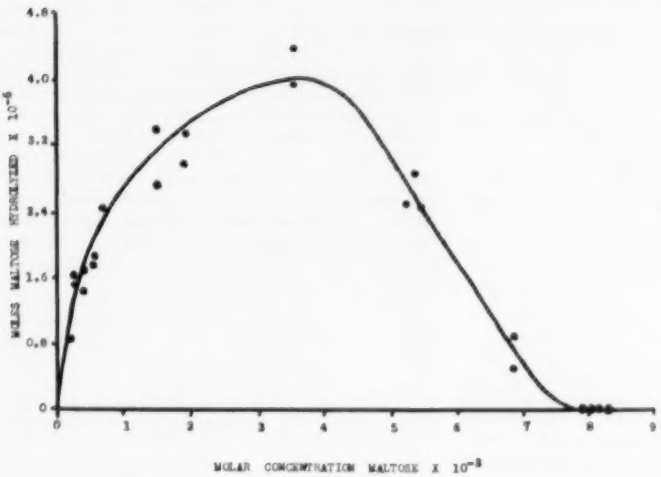


Fig. 3—The effect of substrate concentration on the activity of dog serum maltase. Incubation temperature = 39 C.; period of incubation = 60 minutes; and pH = 6.6.

maltase, if the concentration of maltose is sufficiently high, the activity of maltase may be inhibited or completely blocked. Since maltose hydrolysis may be completely prevented by high concentrations of maltose, and, therefore, no glucose is formed, this inhibitory effect can not be explained solely on the basis of an enzyme-product combination, in this case a maltase-glucose combination. The findings indicate that maltose itself, at high concentrations, inhibits maltase activity.

These data indicate that the maximal hydrolysis of maltose, under the conditions of these experiments, occurs at a maltose concentration of about 3.5×10^{-3} M. Concentrations of maltose greater than this lead to a decrease in the activity of maltase. For this reason, when the maltase activity of dog serum is determined by this method, it is essential that the concentration of maltose in the incubation mixture be less than 3.5×10^{-3} M. Further information concerning a satisfactory maltose concentration to be used in the assay of dog serum for maltase activity was derived from the time-reaction studies.

Time-Reaction Studies.—In order to arrive at a standard incubation time and to study further the effects of maltose concentration on maltase activity, a series of time-reaction studies was prepared. Incubation mixtures containing maltose in the concentrations of 8.13×10^{-3} M, 5.29×10^{-3} M, 1.45×10^{-3} M, and 7.14×10^{-4} M were used; the pH in all mixtures was 6.6. Samples were withdrawn from the incubation mixtures at 15-, 30-, 60-, and 120-minute intervals, and the moles of maltose hydrolyzed were determined. It was found that significant hydrolysis of maltose had taken place in all mixtures by 15 minutes, except in that mixture in which the maltose concentration was 8.13×10^{-3} M; at this concentration, no hydrolysis of maltose was observed throughout the course of the experiment. A typical time-reaction curve obtained from this experiment is shown (fig. 4).

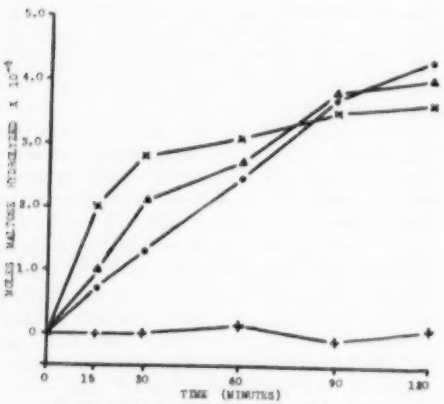


Fig. 4—Time-reaction curve pH = 6.6. Concentrations as follows: + = 8.13×10^{-3} M; × = 5.29×10^{-3} M; ▲ = 1.45×10^{-3} M; and ● = 7.14×10^{-4} M.

In figure 4, the hydrolysis of maltose in the incubation mixture in which the maltose concentration was 7.14×10^{-4} M fol-

lowed nearly a straight line for 120 minutes, at which time 4.3×10^{-6} moles of maltose (75.4%) had been hydrolyzed; moreover, at the 60-minute interval maltose hydrolysis had reached a significant level. This tendency to follow a straight line past the 60-minute interval indicates that at this time maximum hydrolysis had not yet been approached and that the rate of hydrolysis was constant through this time. However, when a maltose concentration of 1.45×10^{-3} M was used, 4.0×10^{-6} moles of maltose (34.5%) were hydrolyzed after 120 minutes and, when the concentration was 5.29×10^{-3} M, only 3.7×10^{-6} moles (8.8%) were hydrolyzed.

The changes in the slopes of those curves obtained when the maltose concentrations were 1.45×10^{-3} M and 5.29×10^{-3} M show an initial rapid hydrolysis followed by a decreasing hydrolytic rate after 30 minutes, although considerable amounts of the substrate, maltose, remain. This decreasing hydrolytic rate indicates that an equilibrium at which hydrolysis is maximum is being approached. That this should occur while the concentration of maltose is yet high is indicative of some inhibitory effect, possibly a maltase-glucose combination acting with the inhibitory effect of high maltose concentrations.

Since the decreasing hydrolytic rate observed with these higher concentrations was not found at a maltose concentration of 7.14×10^{-4} after 60 minutes' incubation, although a significant amount of maltose had been hydrolyzed, this maltose concentration and this incubation time were selected as standard in determining the maltase activity of dog serum.

Maltase Activity in the Serum of the Healthy Dog.—Serum was collected from each of 15 healthy dogs. Using the procedure as described, it was found that at pH 6.6, with a 60-minute incubation at 39 C., 0.01 ml. of dog serum in 8.0 ml. of a 7.14×10^{-4} M maltose solution produced an average hydrolysis of maltose of 1.75×10^{-6} moles. The range was from 1.18×10^{-6} moles to 2.48×10^{-6} moles.

SUMMARY

A method for the assay of maltase activity in the serum of the dog has been described.

The optimal pH of this enzyme was found to lie between pH 6.4 and 6.6, although strong activity was observed between pH 6.1 and 7.3.

The effect of the concentration of the substrate on the activity of dog serum maltase has been discussed. High concentrations of maltose inhibit and may completely block the activity of dog serum maltase.

Information derived from a series of time-reaction studies has been presented and discussed.

Using 15 healthy dogs, it was found that by employing the method of assay described, 0.01 ml. of dog serum hydrolyzed an average of 1.75×10^{-6} moles of maltose in 60 minutes; the range was from 1.18×10^{-6} to 2.48×10^{-6} moles per 60 minutes.

References

[1] Compton, A.: Blood Enzymes. I. On the Occurrence of Maltase in Mammalian Blood. Biochem. J., *15*, (1921): 681–686.

[2] Kokuryo, T.: Untersuchungen über Maltase; über die Blutmaltase. Japan. J. M. Sci. II. Biochem., *2*, (1933): 115–130.

[3] Barnes, B. H., and MacKay, E. M.: The Maltase Activity of the Blood Serum of Various Species. Am. J. Physiol., *114*, (1936): 534–537.

[4] Glock, G. E.: An Investigation of the Rates of Digestion of Starches and Glycogen and the Bearing on the Chemical Constitution. II. Liver Amylase. Biochem. J., *30*, (1936): 2313–2318.

[5] Somogyi, M.: Method for Preparation of Blood Filtrates for Determination of Sugar. J. Biol. Chem., *86*, (1930): 655–663.

[6] Nelson, N.: A Photometric Adaptation of the Somogyi Method for the Determination of Glucose. J. Biol. Chem., *153*, (1944): 375–380.

[7] Kokuryo, T.: Untersuchungen über Maltase; einige Wirkungsbedingungen der Maltase des Blutserums. Japan. J. M. Sci. II. Biochem., *2*, (1933): 161–174.

The Use of a Bacterial Polysaccharide (Piromen) as a Pituitary-Adrenal Stimulant in Dogs

BERNARD C. WEXLER, Ph.D.; JEAN L. FAEHNRICH, B.S.;
MARGARET E. WEISS, B.S.; OLIVER D. GRACE, D.V.M., M.S.

Morton Grove, Illinois

THE RECENT revelation of functional interrelationships between the pituitary and adrenal glands through mediation of adrenocorticotrophic hormone (ACTH) has led to a clearer comprehension of a number of clinical and experimental problems.[1] Because of the remissions induced by ACTH and cortisone in human rheumatoid arthritis, nephritis and nephrosis, dermatomyositis, and in ophthalmological and allergic upheavals, considerable impetus was given to the use of these hormones.[2] Even though the action of these agents produced striking salutary effects, their indiscriminate use was frequently followed by serious complications. Such untoward effects were observed [1, 2] as the masking of fever, depression of the white blood cell count, sodium retention, diabetes in a form unresponsive to insulin, ulceration of the gastrointestinal tract, virilizing effects in the female, and psychotic aberrations. In addition, hasty reduction or withdrawal of the hormones often led to the appearance of the "withdrawal syndrome." This syndrome is characterized by sodium retention followed by severe and precipitous sodium diuresis, by alternation of a wildly euphoric state with one of manic depression, and by sudden elevation of temperature with increases in white blood cells. These complications soon caused the early enthusiasm to wane and the application of ACTH and cortisone was subsequently restricted. More judicious use of these hormones has demonstrated that they may be used safely and effectively when the regimen is so adjusted that optimum dosage levels are maintained to meet the metabolic requirements of the patient.

Experimental[24] and clinical evidence [3, 4, 10, 14, 19, 21, 22, 26] has shown that the bacterial polysaccharide complex, Piromen,* stimulates adrenocortical responsiveness which in many ways mimics that which is caused by ACTH. In the rat adrenal, this drug produces marked alterations in ascorbic acid, cholesterol, and in the histologically demonstrable sudanophilic material.[24] These changes are also attended by lymphopenia, followed by increases in both neutrophils and eosinophils and by augmented liver glycogen deposition. The pattern of change in these entities resembles in many respects that which follows endogenous release of ACTH or its administration. Nevertheless, some striking dissimilarities exist. The maximal fall in adrenal ascorbic acid and cholesterol after a single injection of ACTH usually occurs within one to two hours; after the polysaccharide, this maximal drop is seen in four to five hours. The degree of depletion of ascorbic acid following the administration of this drug is far greater before a plateau is reached than is that which follows ACTH. Prolonged adminis-

TABLE 1—Diurnal Hematological Variations in Normal Dogs (Expressed as Total Cells per Cubic Millimeter x 10^6)

Dog No.		8:00 a.m.	11:30 a.m.	3:30 p.m.
789	w.b.c.	6,125 ± 168	6,300 ± 143	6,350 ± 69
	eos.	71 ± 8	61 ± 6	57 ± 4
	r.b.c.	5.11 ± 0.5	5.09 ± 0.07	5.09 ± 10.0
791	w.b.c.	7,275 ± 92	8,325 ± 245	8,050 ± 39
	eos.	188 ± 15	102 ± 4	138 ± 13
	r.b.c.	5.45 ± 0.004	5.32 ± 0.04	5.13 ± 0.09
792	w.b.c.	6,375 ± 149	6,600 ± 123	6,525 ± 81
	eos.	159 ± 26	116 ± 7	82 ± 4
	r.b.c.	5.67 ± 0.11	5.63 ± 0.04	5.58 ± 0.08
794	w.b.c.	8,475 ± 275	9,025 ± 34	8,850 ± 69
	eos.	116 ± 27	160 ± 15	160 ± 4
	r.b.c.	4.5 ± 0.07	4.34 ± 0.85	4.37 ± 11.0
795	w.b.c.	7,625 ± 210	7,100 ± 75	6,925 ± 75
	eos.	244 ± 15	133 ± 12	121 ± 11
	r.b.c.	5.27 ± 0.07	4.97 ± 13.0	5.1 ± 0.04
796	w.b.c.	8,575 ± 530	7,350 ± 283	8,000 ± 150
	eos.	145 ± 7	184 ± 14	238 ± 11
	r.b.c.	4.14 ± 0.08	4.19 ± 0.05	4.53 ± 0.08
Av. (mean) variation 6 dogs	w.b.c.	7,408	7,450	7,450
	eos.	153	126	132
	r.b.c.	4.99	5.12	5.02

From the Research and Development Department, Baxter Laboratories, Inc., Morton Grove, Ill.

Dr. Wexler's present address is May Institute for Medical Research, Cincinnati, Ohio.

* Piromen is a sterile, nonprotein, and nonanaphylactogenic bacterial polysaccharide complex of Pseudomonas origin. It is prepared as a stable suspension in 1/6 M sodium L-lactate containing 1:10,000 parts of thimerosal. It is a product of Travenol Laboratories, Inc., a subsidiary of Baxter Laboratories, Inc.

TABLE 2—Summary of Hematological Changes in the Dog Produced by a Bacterial Polysaccharide (Piromen) Administered Once per Week for Four Weeks (0.02 Unit per Kilogram of Body Weight *)

No. of dogs		7:00 a.m. 0 hr.	8:00 a.m. 1 hr.	11:30 a.m. 4½ hr.	3:30 p.m. 8½ hr.	8:00 a.m. 24 hr.
			First Week			
6	w.b.c.†	7,408	3,300	7,475	10,540	7,300
	eos.	153	27	60	86	131
	r.b.c.	4.99	4.91	4.96	5.12	5.02
			Second Week			
6	w.b.c.	7,185	3,558	9,758	13,208	7,942
	eos.	160	90	125	211	176
	r.b.c.	5.78	5.49	5.58	5.57	5.46
			Third Week			
6	w.b.c.	10,312	5,506	18,106	22,275	11,112
	eos.	277	144	133	187	261
	r.b.c.	6.35	6.24	6.13	6.24	6.31
			Fourth Week			
4	w.b.c.	13,912	2,712	11,587	24,750	12,800
	eos.	—‡	—	—	—	—
	r.b.c.	—	—	—	—	—

* We selected, as the initial dose of Piromen for the dog, 0.02 unit per kilogram of body weight. In the rabbit and the cat, this amount given intravenously is sufficient to produce an increase in body temperature and a significant alteration in the pattern of blood elements.

† Expressed as total cells per cubic millimeter x 10^6.

‡ No counts were made.

tration of ACTH to the rat causes the adrenal gland to increase in weight; this is accompanied by marked involution of both thymus and spleen and a considerable loss in body weight.[5]

By contrast, the polysaccharide produces an increase in adrenal weight, less marked than that observed after administration of ACTH. The thymus and spleen may either be maintained at a constant size or enlarged. The animal itself continues to grow at an undisturbed rate.[24] Clinically, similar anabolic effects have been observed in human subjects.[4] Increased corticoid output, eosinopenia, and decreased glucose tolerance were among the typical ACTH-like effects produced in healthy male subjects by intravenous injection of relatively large doses (0.5 to 2.0 units†) of this drug. Unlike ACTH, it brought forth a significantly positive balance of nitrogen, with simultaneous retention of both sodium and potassium. The urinary uric acid output was not increased. These effects are most reasonably explained on the basis of growth hormone release by the pituitary gland.

There is a considerable array of veterinary diseases which may be benefited by steroid therapy. In order to determine the potential application of this polysaccharide to this phase of veterinary medicine, we decided to explore the responsiveness of the canine pituitary-adrenal system to this drug.

† For convenience, veterinary Piromen is expressed in terms of units, *i.e.*, 1 unit of veterinary Piromen is equivalent to 50 μg. of Piromen (human).

METHODS

Mongrel dogs of both sexes were selected on the basis of age, weight, health, and disposition. These vigorous and apparently healthy animals were placed in air-conditioned quarters and fed a ration of commercially prepared dog food. All food was withheld during the tests, but water was given *ad libitum*. They were given anti-distemper and rabies vaccine and were tested for worms. The average body weight was 10.7 kg. For the index of adrenal stimulation, the initial fall and succeeding rise of the granulocytes was selected.[15, 20]

Blood samples were taken from the ear in the morning, noon, and midafternoon, in order to verify or disprove the existence of diurnal variations and to establish approximate base-line levels. All counts were made from specimens taken directly from the ear and duplicate counts were made in each instance. For the erythrocyte determinations (erythrocyte counts are expressed as cells per cubic millimeter x 10^6), Hayem's diluting fluid was employed, while eosinophil counts were made according to the method of Randolph.[19] Each sample was shaken for three minutes, plated, and the cells counted. Body temperatures were taken (rectally) at hourly intervals following the injection of the polysaccharide. The maximum rise in temperature occurred three to four hours after the initial injection.

After establishing a hematological base line for the individual animal, a rest period of seven days was allowed before the initial injection and between each succeeding injection. Dosages ranged from 0.001 to 0.6 unit of the drug per kilogram of body weight; all injections were given intravenously.

RESULTS

The data (table 1) show that there was little diurnal variation in the blood ele-

ments. The total counts of leukocytes remained remarkably constant. A small drop in eosinophils was noticed during the noon hour; in man, a slight rise is seen at the same time.[9] The number of red blood cells did not vary throughout the day.

The results of the first trial and successive weekly tests at the same dosage level are compiled (table 2). A precipitous fall in the total number of leukocytes and of eosinophils followed a single injection of 0.02 unit within one hour, while the erythrocyte count remained unchanged. A significant rise in leukocytes and eosinophils appeared in four and a half hours; eight and one-half hours after injection, the leukocytosis was easily discernible, while eosinophils had fallen back to normal levels. All elements had returned to their normal ranges within 24 hours (cf. table 2, fig. 1). Despite these active changes in the blood picture, no increases in body temperature at this dosage level and no untoward reactions were seen.

The polysaccharide was then administered at the same dosage once weekly for three weeks. The same changes in the hematological pattern appeared after each injection. No loss of stimulating effect or no refractoriness was observed. There was, moreover, evidence of an increase in hematopoietic activity in erythrocytic as well as in granulocytic elements (table 2).

Given in dosages ranging progressively from 0.001 to 0.6 unit per kilogram of body weight the drug elicits a graded response in so far as the granulocytic elements of the blood are concerned (cf. table 3, fig. 2). Pyrogenic effectiveness is equally linked to the size of the dose, with the response by increased body temperature lessening as the number of units is decreased. Amounts below 0.02 unit per kilogram of body weight produced no changes in body temperature; maximal temperatures can be induced, however, through the use of larger increments. With heightened temperature, peaks appeared within three to four hours after the injection and the temperature had returned to normal in eight hours. Leukopenia was seen earlier—in one to two hours after the injection. While a good response was invoked by 0.01 to 0.02 unit, the reduction in leukocytes was more frankly exhibited at higher levels of medication. Leukocytosis of unusual intensity, delayed both in the attainment of maximal levels (to 24 hours) and in its disap-

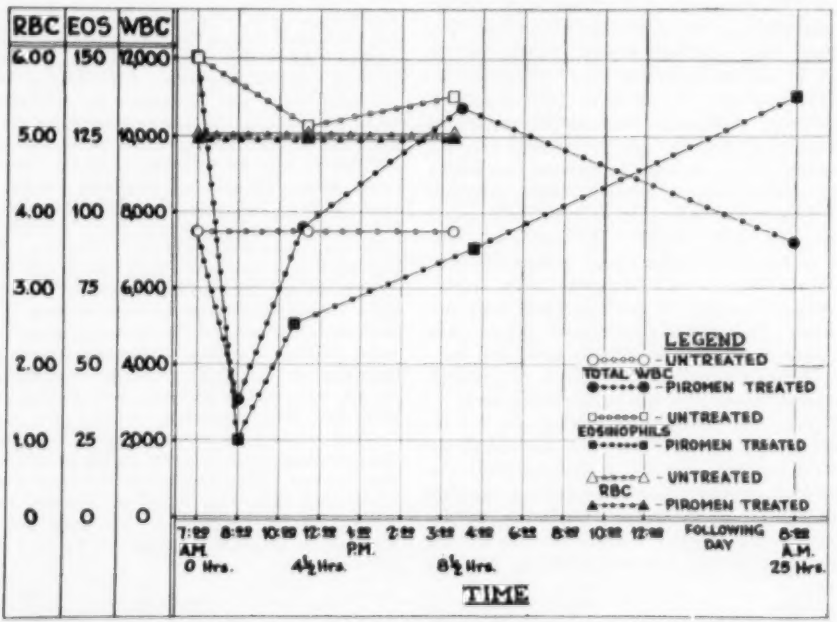


Fig. 1—The effect of Piromen (0.02 units/kg., intravenously) on various hematological elements in the dog.

TABLE 3—Average Temperature and Total White Blood Cell * Response to Graduated Doses of Piromen (Single Injection) in the Dog (0.001 to 0.6 Unit per Kilogram of Body Weight, Intravenously)

Dose (units)	No. of dogs	Temperature rise (3 to 4 hr.)	0 hr.	1 hr.	2 hr.	4 hr.	6 hr.	8 hr.	24 hr.	48 hr.	96 hr.
0.6	4	3.77	14,250	5,875	5,275	9,187	18,950	†	49,062	†	15,200
0.4	2	3.63	12,675	6,175	4,500	9,750	15,750	18,050	46,650	†	13,025
0.2	3	3.42	11,566	7,000	5,266	7,516	13,700	19,250	32,650	†	10,186
0.1	4	2.73	10,612	8,725	2,462	5,927	7,637	†	16,500	10,500	—
0.06	2	3.8	18,175	6,400	8,500	25,950	24,600	†	27,475	17,615	—
0.04	2	1.45	16,025	6,525	7,850	23,100	33,100	†	27,825	18,000	—
0.02	37	0	5,151	2,206	6,861	5,107	12,496	18,260	11,260	6,101	—
0.01	11	0	12,881	7,959	8,222	9,909	12,577	14,593	14,118	13,464	—
0.002	15	0	11,003	10,332	11,683	12,390	13,586	14,908	13,543	10,090	—
0.001	10	0	14,635	14,840	13,560	13,310	14,690	14,935	14,760	—	—

* Expressed as cells per cubic millimeter x 10^6.
† Count not taken.

pearance (after 96 hours), followed the administration of 0.1 to 0.6 unit.

DISCUSSION

In formulating an index of adrenocortical function or reserves, the use of the changing levels in circulating eosinophils in the dog has been suggested by Martin *et al.*[15] who found that the response to ACTH, as shown by eosinophilic elements, is essentially the same in the normal dog as in man. A fall of 70 per cent or more

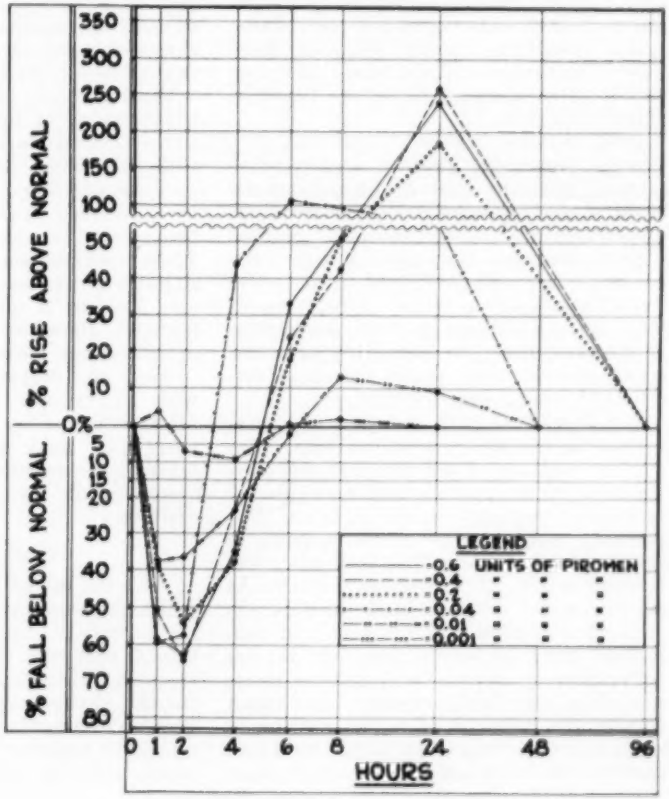


Fig. 2—Time response of total white blood cells in the dog to a single intravenous injection of Piromen (0.001 to 0.6 units/kg.).

in circulating eosinophils within seven hours after a test dose of ACTH is presumed to indicate satisfactory adrenocortical reserves. This diagnostic procedure, like all clinical tests, must be correlated with other findings and, also, with other more critical tests.

While our findings agree with those reported by Martin, we have found the granulocytopenic, agranulocytopic, and granulocytophilic indexes equally reliable, and would suggest that these be used in conjunction with the eosinophil test.

It is of interest that, after a single test dose of the polysaccharide, eosinopenia, like leukopenia, appears within one hour. According to Martin, a single dose of ACTH does not produce a maximal fall in the eosinophil count for seven hours.[15] The decline in both eosinophils and leukocytes after a test dose of Piromen was 58 per cent; this response is less than that which follows administration of ACTH.

While the eosinophil count did not exceed normal levels, counts 80 per cent above levels were recorded for leukocytes. Leukocyte counts reaching three times the normal ranges have been reported after large doses of the bacterial polysaccharide.[25]

Data collected (table 1), and that previously reported,[15] present corroborative evidence that the formed elements in the blood of the dog normally differ but little throughout the day. This fact, coupled with a sensitive response of these elements to the polysaccharide or to ACTH, should encourage a wider clinical use of these indexes as diagnostic aids, suggesting, at the same time, the possibility of wider application of these potent agents in veterinary medical practice.[16]

The alterations in the numbers of granulocytes in the blood, reported here, offer further evidence of the pituitary-stimulating effect of the polysaccharide, *i.e.*, stimulation of endogenous release of ACTH. The intensity and character of the response is less pronounced than that seen after the administration of exogenous ACTH.[15] As stated earlier, experimental animals exhibiting decreased ascorbic acid and cholesterol after the polysaccharide [24] did not suffer loss of thymus and splenic tissue nor show the body weight loss which follows the injection of ACTH. In human subjects,[4] this drug induces the metabolic changes and increased adrenal output characteristic of ACTH release and, unlike the hormone, positive nitrogen retention.

The increases in erythrocytic and granulocytic elements observed when the polysaccharide was given at weekly intervals (table 2) could be attributed to the induced release of adrenocorticoids which are known to be potent hematopoietic agents.[7, 11] Elevations in the numbers of leukocytes after larger amounts were administered resemble those encountered in the results reported by Windle and Wilcox.[25] In these studies, the ability of the drug to stimulate the reticuloendothelial system was measured; this stimulation is, likewise, a known effect of adrenocorticoids.[11] The polysaccharide has been used with considerable success for certain allergic dyscrasias, such as neurodermatitis, and in ophthalmological disorders.[6, 12, 13, 17, 25] This may be due to its capacity to stimulate the reticuloendothelial system.

No evidence of developing refractoriness has been found after repeated weekly injections of 0.02 unit (table 2). Conn [4] observed that, unlike ACTH and typhoid vaccine, it induced adrenal response in human subjects even after repeated injection.

Finally, none of the untoward effects which so often follow administration of adrenocortical factors were encountered with this drug. In dogs, these may take the form of edema, polydipsia, or polyuria. In man, they appear as transient chills, fever, rectal tenesmus, and general malaise. These reactions may be avoided by proper adjustment of the dose. In both dogs and cats, 1.0 unit or more may produce vomiting and illness shortly after intravenous injection, but recovery is usually prompt.

Since the polysaccharide can be given with safety and without a disturbing aftermath, it may come to be regarded, eventually, as a useful agent for stimulating adrenocortical activity in the alleviation of the toxic effects of many animal diseases.[6]

SUMMARY

With the use of leukopenia, eosinopenia, and leukocytosis as indexes of adrenocortical activity in the dog, evidence was obtained that the bacterial polysaccharide complex, Piromen, is a potent stimulant for the pituitary-adrenal system. The degree of stimulation, when measured by the leukocyte response, showed a gradient proportional to the administered dose. Intra-

venous injection of 0.01 to 0.02 unit per kilogram of body weight elicited marked alteration in the circulating granulocytic elements; this activity was not accompanied by pyrogenic effects. Administration of 0.02 unit at weekly intervals did not lead to the development of refractoriness of response. Above such dosage levels, there was a gradual increase in body temperature in response to each increment, with intensified hematological response. The injection of 0.2 unit of this polysaccharide brought significant stimulation of the reticuloendothelial system. The absence of untoward effects suggests that the drug may have promise as a safe means for stimulating pituitary-adrenal activity in animals.

References

[1] Sprague, R. G.: Cortisone and ACTH: A Review of Certain Physiologic Effects and Their Clinical Implications. Am. J. Med., *10*, (1951): 567.

[2] Mote, J. R.: Proceedings of the Second Clinical ACTH Conference. Blakiston Co., New York, N. Y., 1951.

[3] Arendhorst, W., and Falls, H. F.: Role of the Adrenal Cortex in Treatment of Ocular Diseases with Pyrogenic Substances. Am. M. A. Arch. Ophth., *44*, (1950): 635.

[4] Conn, J. W., Fajans, S. S., Louis, L. H., Seltzer, H. S., and Kaine, H. D.: A Comparison of Steroidal Excretion and Metabolic Effects Induced in Man by Stress and by ACTH. Recent Progress in Hormone Res., *10*, (1954): 471.

[5] CoTui, F. W., and Schrift, M. H.: Production of Pyrogen by Some Bacteria. J. Lab. and Clin. Med., *27*, (1942): 569.

[6] Dakin, W. V., and Au, R. J.: Piromen Therapy in Dogs. Calif. Vet., *7*, (1954): 26.

[7] Daughaday, W. H., Williams, R. H., and Daland, G. A.: The Effect of Endocrinopathies. Blood, *3*, (1948): 1342.

[8] Dougherty, T. F., and White, A.: Influence of Hormones on Lymphoid Tissue Structure and Function. The Role of Pituitary Adrenotrophic Hormone in Regulation of the Lymphocytes and Other Cellular Elements of the Blood. Endocrinology, *35*, (1944): 1.

[9] Forsham, P. H., Thorn, G. W., Prunty, F. T. G., and Hills, A. G.: Clinical Studies with Pituitary Adrenocorticotropin. J. Clin. Endocrinol., *8*, (1948): 15.

[10] Forsham, P. H.: Unpublished observations, 1954.

[11] Gordon, A. S., and Charipper, H. A.: The Endocrine System and Hemopoiesis. Ann. New York Acad. Sci., *48*, (1947): 615.

[12] Kaumeyer, W. F.: Unpublished observations, 1950.

[13] Kierland, R. R., and Kulwin, M. H.: Clinical Evaluation of a New Pyrogenic Agent. Am. M. A. Arch. Dermat. and Syph., *62*, (1950): 571.

[14] Kirkendall, W. M., Hodges, R. E., and January, L. E.: The ACTH-like Effect of Fever in Man. J. Lab. and Clin. Med., *36*, (1950): 845.

[15] Martin, J. E., Skillen, R. G., and Deubler, M. J.: The Action of Adrenocorticotrophic Hormone on Circulating Eosinophils in Dogs. A Proposed Screening Method for Evaluating Adrenal Cortical Function. Am. J. Vet. Res., *15*, (1954): 489.

[16] Martin, J. E., LaGrange, W. E., Fielder, F. G., Skelley, J. F., and Arnold, M. W.: The Use of Cortisone and Hydrocortisone in Veterinary Medicine. Proc. Book, AVMA (1954): 115–119.

[17] McCorriston, L. D.: Piromen Treatment of Various Dermatoses. Canad. M.A.J., *68*, (1953): 137.

[18] Randolph, T. G.: Differentiation and Enumeration of Eosinophils in the Counting Chamber with a Glycol Stain: A Valuable Technique in Appraising ACTH Dosage. J. Lab. and Clin. Med., *34*, (1949): 1696.

[19] Randolph, T. G., and Rollins, J. P.: Pyromen in the Treatment of Perennial Allergic Symptoms. Ann. Allergy, *8*, (1950): 626.

[20] Reinhardt, W. O., Aron, H., and Li, C. H.: Effect of Adrenocorticotrophic Hormone on the Leukocyte Picture of Normal Rats and Dogs. Proc. Soc. Exptl. Biol. and Med., *57*, (1944): 19.

[21] Samter, M., and Kofoed, M.: On the Rationale of Treating Allergic Diseases with Bacterial Pyrogens. J. Allergy, *23*, (1952): 327.

[22] Samuels, L. T., and Eik-Nes, K.: Unpublished observations, 1954.

[23] Sayers, G.: The Adrenal Cortex and Homeostasis. Physiol. Rev., *30*, (1950): 241.

[24] Wexler, B. C.: Unpublished observations, 1954.

[25] Windle, W. F., and Wilcox, H. H.: Extramedullary Hemopoiesis in the Rabbit and Cat Induced by Bacterial Pyrogens. Am. J. Physiol., *163*, (1950): 762.

[26] Wittich, F. W.: Pyromen in the Treatment of Perennial Respiratory Allergies. Ann. Allergy, *9*, (1951): 502.

Symposium on Immunization Against Infectious Bronchitis Virus. I. Some Basic Properties of Infectious Bronchitis Virus

CHAS. H. CUNNINGHAM, D.V.M., Ph.D.

East Lansing, Michigan

THE PURPOSE of the present contribution is to bring together some of the presently known properties of the virus of infectious bronchitis (IB) of chickens. The clinical features, methods of diagnosis, and control of the disease have been adequately described.[1,5,8,17,18,24-26,44,48,52,67-69] Reference will be made to these facets only where significant for introduction of the basic properties of the virus.

One of the difficulties in assessment of the properties of infectious bronchitis virus (IBV) is the lack of definitive characterization of strains employed in many studies. Fundamentally, virology should be concerned primarily with the properties of the agent for interpretation of the changes induced in the host, directly or indirectly, in terms of mechanisms of the virus-cell interaction. Research findings should serve as a stimulus for more penetrating studies of IBV by this avenue of approach.

CLASSIFICATION

Classification of IBV in the order Virales as *Tarpeia pulli* was first proposed by Packer[56] and has been continued in van Rooyen's[70] revision of Suborder III, Zoophagineae, viruses infecting animals. Infectious bronchitis virus is included in Family VII, Reedellaceae fam. nov., viruses causing generalized systemic infection characterized by fever and necrosis of tissue in the absence of obvious macule, papule, or vesicle formation or of conspicuous involvement of nerve cells, and Genus III, Tarpeia, viruses of the influenza group inducing diseases characterized principally by involvement of the respiratory tract.

From the Department of Microbiology and Public Health, Michigan State University, East Lansing. Journal article 1966 of the Michigan Agricultural Experiment Station.

Presented before the Section on Poultry, Ninety-Third Annual Meeting, American Veterinary Medical Association, San Antonio, Texas, Oct. 15-18, 1956. Part III of the symposium appears in the April 15, 1957, issue of the JOURNAL of the AVMA.

MORPHOLOGY

Infectious bronchitis virus in allantoic fluid is spherical, averaging from 60 to 100 mμ, with filamentous projections which are more prominent when specimens are prepared in saline than in distilled water.[59,61,62]

In chorioallantoic membranes, intracytoplasmic elementary bodies appear as spheres averaging 200 mμ.[23] Smaller particles and doughnut-shaped forms which sometimes possess evident internal structure may be observed. In some specimens, the tissues appear almost normal with the elementary bodies enmeshed separately, in pairs, chains, or clumps of varying sizes. In severely affected tissue, the cytoplasmic network is obscured by a dense and seemingly mucoid material which is observed as the imbedding matrix of the viral clumps and as thin strands between individual particles and tissue elements.

HOST SPECTRUM

Chickens.—The chicken is the natural host for IBV and, as far as known, other avian species are not affected.[67] Infection may be induced by several routes of inoculation[17] but the intratracheal route is most commonly employed for artificial transmission.[28,40,41,43,44,47,48] Following intratracheal instillation of the virus, the tracheal mucosa undergoes cyclic changes in three main phases for 18 to 21 days: (1) acute phase (1 to 3 days)—epithelial hypertrophy and marked edema, (2) reparative phase (6 to 9 days)—epithelial hyperplasia and marked cellularity of the propria, and (3) immune phase (12 to 18 days)—restoration of the epithelium and either follicular or mild focal, diffuse lymphoid infiltration of the propria.[47]

Significant changes are not seen in histological sections of the liver, spleen, and kidney. Inclusion bodies are not observed.[40]

The virus can be isolated readily from lung and tracheal material during the in-

cubative and throughout the respiratory phases of the disease which may vary from 24 hours to as long as four weeks after infection.[17,24,28,41,43] The virus may also be isolated from eggs from two to 43 days after infection.[28]

The onset of symptoms, histological changes, and virus-neutralizing antibodies are primarily conditioned by virus dosage. Low doses of virus, 0.1 to 1.0 minimum embryo infective dose (m.e.i.d.), have a delaying effect and high doses, 10^2 to 10^6 m.e.i.d., have an accelerating effect. The chicken appears to be more susceptible to comparable doses of IBV than the chicken embryo.[47]

Embryonating Chicken Egg.—Infectious bronchitis virus can be propagated readily in embryonating chicken eggs via several routes of inoculation. On primary isolation, gross pathological alterations of the embryo are employed as evidence of viral activity. Embryo mortality is not a constant finding.[2,8,11,15,17,19,21,24,27] Among the alterations considered most typical are "curling" of the embryos with a wry neck and deformed feet compressed over the head. Embryos may be dwarfed as much as one-half size with a firm, ball-like shape.[24,52] Living embryos are sluggish in their movements. The amnionic fluid is considerably less than normal and the membrane is thickened, dry, and fibrotic. Other alterations are edema and necrotic foci of the kidneys, dermal petechiation, and distension of the cloaca with fatlike droplets.[52]

Microscopic findings consist of edema of the chorioallantoic membrane and proliferation of the mesodermal and ectodermal cells. Other alterations are hepatic hemorrhage, necrosis, and abscess formation; pneumonia with marked serous exudation; and interstitial nephritis and necrosis. Inclusion bodies are not observed.[52]

Following inoculation via the allantoic cavity, amnionic cavity, and a modified chorioallantoic membrane method (inoculum introduced onto the shell membrane at the base of the air cell and into the allantoic cavity), gross pathological alterations may be observed on primary isolation.[8,11,19,24,27,52] With the conventional chorioallantoic membrane method, several passages may be necessary before these changes are evident.[2,17,21] Inoculation via the yolk sac may be successful if the embryo does not contain maternal antibodies.[11,49] While embryo mortality is not a constant finding on initial passage, the highest rate is produced from inoculation via the amnionic cavity followed in decreasing order by the allantoic cavity and chorioallantoic membrane methods.[11] The allantoic cavity[8,11,19,24,52] and modified chorioallantoic membrane methods[27] are the most desirable for initial isolation of IBV with some advantage claimed for the latter in detection of viral activity in earlier embryo passage.[27]

Serial passage of IBV in embryonating chicken eggs is accompanied by an increase in virulence of the virus for embryos and a decrease in virulence for chickens paralleled by loss of antigenicity and immunogenicity.[2,21] Embryo-cultivated virus still capable of infecting chickens does not regain its original characteristics on reisolation in embryos.[21] Completely modified or embryo-adapted virus will kill all embryos within 24 to 36 hours. These embryos seldom show signs of infection other than dwarfing and congestion and swelling of the kidneys with necrotic foci.[28] Although embryo-adapted virus is nonantigenic, it is specifically neutralized by anti-IB serum.[28,57,58]

Cell (Tissue) Culture.—The virus can be cultivated in serial passage in minced chorioallantoic membrane in medium 597 (medium 199 from which purines and pyrimidines have been omitted and containing Hank's solution) either alone or supplemented with normal horse serum or chicken embryo extract. Virus is produced as long as cellular growth occurs. Cytopathogenic effects and change of pH of infected cultures are not observed. Chicken embryo and monkey kidney cells support growth of IBV, but mouse or rat liver cells do not.[29]

Infectious bronchitis virus may also be cultivated in the entire chorioallantoic membrane in Tyrode's solution with change of pH of the infected culture.[12]

Miscellaneous.—Clinical signs of infection are not evident in rabbits[64] and Swiss mice[50,66] inoculated with IBV by the intranasal and other routes. A slight pulmonary congestion may be observed in mice but this is not a significant or constant finding.[66] Serial passage of the virus in mice is not successful. It apparently does not infect but merely survives in mouse lung following intranasal instillation. Alternate

embryo-mouse-embryo passage is possible with embryo-adapted virus if specimens are collected at a 12-hour postinoculation period from the mice.[56]

The cynomolgus monkey *(Macaca cynomulgus)* and the cave bat *(Myotus lucifugus)* are considered susceptible to IBV.[16,63]

Growth Rates

Infectious bronchitis virus propagates rapidly in, and invades, all tissues of the embryonating chicken egg.[10,11,21,38] There is no significant difference in the final virus concentration with different dilutions of inoculum via the allantoic cavity, but the time necessary for the virus to go into the log phase and reach maximum concentration is directly correlated with the concentration of the inoculum. With early passage IBV, the log phase of growth occurs at six hours postinoculation and maximum concentration is attained within 24 to 30 hours. Slight decreases occur at 36, 42, and 48 hours. Embryo-adapted virus is in the log phase in four hours with maximum concentration of the virus occurring at 12 hours.[38]

Following inoculation of embryonating chicken eggs with IBV and isotope P^{32}, essentially no differences are observed in the distribution of the isotope in the embryo through the eleventh hour of incubation. Beginning at the twenty-fourth hour, the activity of the isotope in the vrus-infected allantoic fluid is less than that in control eggs. A marked concentration of P^{32} occurs in the embryo minus liver, embryo liver, and chorioallantoic membrane 24, 48, and 72 hours after inoculation. Similar results[50] are obtained with S^{35}.

In cell (tissue) culture, an absorption period with complete disappearance of virus occurs from four to eight hours after inoculation. The latent period for early passage virus is 12 to 16 hours and new virus is formed for at least three days. The latent period for embryo-adapted virus is eight to 12 hours and new virus is formed for at least eight days.[29]

Assay of Viral Infectivity

Viral infectivity may be assayed quantitatively by serial dilution through interpolation of the 50 per cent end point of cumulative frequencies of positive and negative responses of the indicator host.[9,65] Chickens and embryonating chicken eggs may be employed but the latter are more frequently used because of simplicity of interpretation and ease of control of infection.

The virus in early embryo passage does not kill embryos consistently and additional evidence of viral infectivity such as typical gross pathological alterations of living embryos on the sixth to eighth days postinoculation must be included.[38] With completely egg-adapted virus, mortality rates alone may be used as the criterion of infectivity.[9]

Comparable accuracy in titration of viral infectivity, $\sigma = 10^{0.07}$, and infectivity neutralization, $\sigma = 10^{0.2}$, of embryo-adapted virus may be attained using five, seven, eight, or ten eggs per decimal dilution.[58]

The virus does not possess hemagglutinative activity.[25,42]

Serology

Antigenicity of IBV and the production of neutralizing antibodies is directly associated with infectivity of the virus for chickens.[20,21,46] Inactivated virus and completely modified or embryo-adapted virus do not possess these properties. Antibody response can be assayed quantitatively by the neutralization test employing either the antigen-dilution[9,57,58] or serum-dilution[6] techniques. The lethal $dose_{50}$ neutralization index ($l.d._{50}NI$) of 99.7 per cent of normal chicken serums with the virus dilution technique would not be expected to exceed $10^{1.517} \pm 10^{0.0376}$, or approximately 36 neutralizing doses.[7]

Decimal dilution of anti-IB serum follows a linear exponential regression when either phosphate buffer, 0.85 per cent NaCl or nutrient broth are used as diluents for the viral infectivity neutralization test.[58]

From 84.0 to 91.0 per cent of the infectivity neutralizing potential of anti-IB serum is utilized immediately on contact with virus. Maximum neutralization occurs within 15 to 20 minutes at 4 C., 22 C., or 37 C. and no increase occurs with prolonged incubation. Anti-IB serum stored at 4 C. for eight weeks or at 22 C. for seven days prior to testing does not decrease in neutralizing capacity. After storage at 37 C. for 56 hours, a tenfold decrease occurs.[58]

Neutralizing antibodies are detectable and measurable from about the second week following primary antigenic stimulus

of adult chickens with virulent virus with the maximum level being attained at about the sixth week.[22, 57, 58] The level remains relatively constant through the twelfth week followed by a decline at the twentieth week. Secondary antigenic stimulus at the twelfth week is manifested by a further increase in neutralizing antibodies for about three weeks with a slight decline at the twentieth week. At this time, the level is above the maximum induced by primary stimulus at the twelfth week.[22]

Information as to the residence and function of antibodies within certain serum components must await further study. Electrophoretic analysis by the Tiselius method shows that primary antigenic stimulus is accompanied by a marked but transient increase of total globulin as influenced by an increase of *gamma* globulin within two weeks. This is followed by a recession to minimum levels. Secondary antigenic stimulus is not accompanied by a significant increase of globulin. Other than the brief initial relationship, neutralizing antibodies are not quantitatively associated with increased levels of globulin. Neutralizing antibody formation is not impaired by imbalance of serum components from protein depletion produced by withdrawal of large amounts of blood at weekly intervals following primary antigenic stimulus.[22]

Spectrophotometric analysis of chemically fractionated serum indicates that *alpha* and *beta* globulins may also be associated in some manner with primary antigenic stimulus and neutralizing antibodies.[37]

Interference

Interference occurs between active and heat-inactivated IBV in embryo culture. An interfering material may also be demonstrated in infected allantoic fluid from dead embryos stored for longer than two hours at 36 C.[31, 36]

In embryo culture, maximum interference occurs between IBV in early egg passage challenged later by influenza type B virus. Less interference takes place between embryo-adapted IBV and influenza type A virus challenged by early egg passage IBV. Simultaneous injection of early passage and embryo-adapted IBV, or challenge of influenza type A virus by early passage IBV, is without effect.[32]

Infectious bronchitis virus can be propagated simultaneously with Newcastle disease virus (NDV) in eggs but the action of IBV is masked by the more lethal action of NDV.[34] As the concentration of NDV is decreased in the presence of undiluted IBV in embryo culture, the hemagglutinative activity of NDV becomes irregular. Infectious bronchitis virus propagates well irrespective of the concentration of NDV. The m.e.i.d. of NDV is not altered by varying concentrations of IBV.[33] Active immunity develops to both viruses, and antiserums satisfactorily neutralize the homologous virus in dual infections, thus permitting demonstration of the presence of each virus.[34, 55] Interference with the development of signs of Newcastle disease may be induced by IBV following infection of chickens with both viruses, particularly when IBV is in excess but to a lesser degree when NDV is in excess.[35, 39]

Demonstration of interference between these viruses does not necessarily imply taxonomic relationship but it may indicate a common pathway of infection within the host cell.[36]

Environmental Influences

Heat.—Thermal inactivation of embryo-adapted IBV is exponential with the activation energy for inactivation being of the approximate order of 24,000 calories/mole at 4 C. Rates of inactivation are as follows: 4 C., $10^{0.26}$/week; 22 C., $10^{0.30}$/day; 37 C., $10^{0.14}$/hour; and 56 C., $10^{1.18}$/minute.[58]

Most strains of IBV are inactivated at 56 C. in ten minutes. The thermal stability of different strains may be detected after exposure at 56 C. for intervals of 15, 30, and 45 minutes. There appears to be a tendency toward increased thermal susceptibility of the virus after continued passage in embryos. Horse serum incorporated in the diluent has a protective influence on the virus.[45]

Dehydration.—The virus is more stabile in the lyophilized state than in the liquid state.[3-5] Data as to maximum viability in the lyophilized state as determined by embryo inoculation and chicken inoculation, respectively, are as follows: —25 C., at least 606 and 378 days; 4 C., 500 and 260 days; 20 to 30 C., 458 and 269 days; and 37 C., 106 and 14 days. Maximum viability of the virus in allantoic fluid as deter-

mined by embryo inoculation is as follows: —25 C., 537 days; 4 C., 427 days; 20 to 30 C., 64 days; and 37 C., 3 days.

One strain of IBV lyophilized in a desiccator over P_2O_5 and stored under these conditions has been found active after 3,077 days.[3]

Radiation.—Ultraviolet light is without effect on IBV.[51]

pH.—The stability range of IBV infectivity at 4 C. is initially greater in an acid medium (pH 3.0 for 14 days) than in an alkaline medium (pH 10.6 for 2 days), with maximum stability at pH 7.8 for 170 days.[14]

The isoelectric point of the virus is perhaps at pH 4.05.[14]

Chemical Agents.—Embryo-propagated IBV is inactivated within three minutes by such agents as 1 per cent phenol, 1 per cent liquor cresolis saponatus, 1:10,000 solution of potassium permanganate, 1 per cent tincture of metaphen, 25 per cent ethyl alcohol, 1:1,000 mercuric chloride, 1 per cent formalin, 5 per cent sodium hydroxide, and 1:1,000 tincture of zepharin.[13]

Carboxide (10% ethylene oxide and 90% carbon dioxide) is without effect on lyophilized IBV during exposure for 16 hours. In the moist state, the virus is inactivated within four hours. Ethylene oxide is considered as the virucidal agent of carboxide in the concentration of 400 mg. per liter.[54]

Antibiotic Agents.—Penicillin and streptomycin are without effect on IBV.

Viscosin, prepared from *Pseudomonas viscosa,* exerts a highly protective effect on embryos when introduced one hour before infection with IBV. When introduced one hour after infection, the effectiveness is markedly diminished but not completely abolished. At four hours after infection, viscosin is without significant effect.[33]

Miscellaneous.—Polylysine exerts a marked protective effect on embryos subsequently infected with IBV in early egg passage. This effect is significantly less with virus after several egg passages and with virus which is completely egg-adapted. The resistance to polylysine inhibition with increased egg passage of the virus may be due to (1) a reflection only of an increase in virulence of the virus for embryos, or (2) a modification of the surface pattern of the virus in such a manner as to render it less available for combination with polylysine. It is suggested that polylysine inhibits IBV by a combination of basic polypeptide with acidic groups on the surface of the virus protein.[30]

Protamine shows a slight amount of protection of embryos against infection with IBV and there is little or no effect by lysozyme.[30]

SUMMARY

1) Infectious bronchitis virus (IBV) is classified as *Tarpeia pulli* in Order Virales.

2) The virus is spherical, averaging from 60 to 100 mμ in allantoic fluid and 200 mμ in the chorioallantoic membrane where it is intracytoplasmic. Inclusion bodies are not observed.

3) The chicken is the natural host for IBV. Following intratracheal instillation of the virus, the tracheal mucosa undergoes cyclic changes in three main phases (acute, reparative, and immune) for 18 to 21 days. The virus can be propagated in embryonating chicken eggs and cell (tissue) culture. The rabbit and Swiss mouse are not suitable hosts. The cynomolgus monkey *(Macaca cynomolgus)* and the cave bat *(Myotus lucifugus)* are considered susceptible to IBV.

4) Neutralizing antibodies are produced in chickens with virulent IBV. A brief but transient relationship exists between globulins and neutralizing antibody following primary antigenic stimulus.

5) Comparable accuracy in quantitative determination of viral activity may be attained with five, seven, eight, or ten embryonating chicken eggs per decimal dilution.

6) Decimal dilution of anti-infectious bronchitis (IB) serum is exponential in assay of neutralizing antibody. From 84 to 91 per cent of the infectivity neutralizing potential of anti-IB serum is utilized immediately on contact with the virus. Maximum neutralization occurs within 15 to 20 minutes.

7) Interference occurs between active and heat-inactivated IBV, influenza, and Newcastle disease viruses.

8) Thermal inactivation of IBV is exponential. The virus is more stabile in the lyophilized state than in the liquid state. The chemical agents commonly used for disinfection inactivate IBV. Ultraviolet light is without effect on the virus for the control of the natural transmission of the disease.

9) Penicillin and streptomycin are without effect on IBV. Viscosin and polylysine are effective under certain conditions.

References

[1] Beaudette, F. R.: Twenty Years of Progress in Immunization Against Virus Diseases of Birds. J.A.V.M.A., *115*, (1949): 367–377.

[2] Beaudette, F. R., and Hudson, C. B.: Cultivation of the Virus of Infectious Bronchitis. J.A.V.M.A., *90*, (1937): 51–58.

[3] Beaudette, F. R., Miller, B. R., Bivins, J. A., and Hudson, C. B.: The Viability of Dried Viruses of Avian Origin. Am. J. Vet. Res., *9*, (1948): 190–194.

[4] Billings, S. A.: The Effect of Sublimation and Storage on an Egg-Adapted Strain of Infectious Bronchitis Virus. M.S. thesis, Michigan State Univ., 1956.

[5] Brandt, C. D., Van Roekel, H., and Peck, H. A.: An Egg-Propagated Immunizing Agent for the Control of Infectious Bronchitis of Chickens. Poult. Sci., *31*, (1952): 1004–1008.

[6] Crawley, J. F.: The Measurement of Antibodies to Chick Bronchitis Virus by Use of the Serum Dilution Technique. Proc. Northeast. Conf. Lab. Workers Pullorum Dis. Control, Univ. Connecticut, Storrs, 1951.

[7] Cunningham, C. H.: Newcastle Disease and Infectious Bronchitis Neutralizing Antibody Indexes of Normal Chicken Serum. Am. J. Vet. Res., *12*, (1951): 129–133.

[8] Cunningham, C. H.: Methods Employed in the Diagnosis and Investigation of Infectious Bronchitis and Newcastle Disease. Proc. Book, AVMA (1952): 250–257.

[9] Cunningham, C. H.: A Laboratory Guide in Virology. Burgess Publishing Co., Minneapolis, 1956.

[10] Cunningham, C. H., and El Dardiry, A. H.: Distribution of the Virus of Infectious Bronchitis of Chickens in Embryonated Chicken Eggs. Cornell Vet., *38*, (1948): 381–388.

[11] Cunningham, C. H., and Jones, M. H.: The Effect of Different Routes of Inoculation on the Adaptation of Infectious Bronchitis Virus to Embryonating Chicken Eggs. Proc. Book, AVMA (1953): 337–342.

[12] Cunningham, C. H., and Spring, M. P.: Unpublished data.

[13] Cunningham, C. H., and Stuart, H. O.: The Effect of Certain Chemical Agents on the Virus of Infectious Bronchitis of Chickens. Am. J. Vet. Res., *7*, (1946): 466–469.

[14] Cunningham, C. H., and Stuart, H. O.: The pH Stability of the Virus of Infectious Bronchitis of Chickens. Cornell Vet., *37*, (1947): 99–103.

[15] Cunningham, C. H., and Stuart, H. O.: Cultivation of the Virus of Infectious Bronchitis of Chickens in Embryonating Chicken Eggs. Am. J. Vet. Res., *8*, (1947): 209–212.

[16] Delaha, E. C., Reagan, R. L., Cook, S. R., and Brueckner, A. L.: Transmission of the Avian Infectious Bronchitis Virus to the Cynomolgus Monkey. Cornell Vet., *44*, (1954): 433–436.

[17] Delaplane, J. P.: Studies of Infectious Bronchitis. Rhode Island State Coll., Agric. Exper. Sta. Bull. 273, 1939.

[18] Delaplane, J. P.: The Differentiation of the Respiratory Diseases of Chickens. Rhode Island State Coll., Agric. Exper. Sta. Bull. 288, 1943.

[19] Delaplane, J. P.: Technique for the Isolation of Infectious Bronchitis of Newcastle Virus Including Observations on the Use of Streptomycin in Overcoming Bacterial Contaminants. Proc. Northeast. Conf. Lab. Workers Pullorum Dis. Control, North Carolina State Coll., Raleigh, 1947.

[20] Delaplane, J. P.: Progress in Infectious Bronchitis Research. J.A.V.M.A., *115*, (1949): 257–258.

[21] Delaplane, J. P., and Stuart, H. O.: The Modification of Infectious Bronchitis Virus of Chickens as the Result of Propagation in Embryonated Chicken Eggs. Rhode Island State Coll., Agric. Exper. Sta. Bull. 284, 1941.

[22] Dimopoullos, G. T., and Cunningham, C. H.: Electrophoretic and Serum-Neutralization Studies of Serums from Chickens Exposed to Infectious Bronchitis Virus. Am. J. Vet. Res., *17*, (1956): 755–762.

[23] Domermuth, C. H., and Edwards, O. F.: Electron Microscope Observations of Thin-Sections of Chorio-Allantoic Membranes Infected with the Virus of Avian Infectious Bronchitis. Proc. Soc. Am. Bact. (1954): 71–72.

[24] Fabricant, J.: Studies on the Diagnosis of Newcastle Disease and Infectious Bronchitis. II. The Diagnosis of Infectious Bronchitis by Virus Isolation in Chick Embryos. Cornell Vet., *39*, (1949): 414–431.

[25] Fabricant, J.: Studies on the Diagnosis of Newcastle Disease and Infectious Bronchitis of Fowls. III. The Differential Diagnosis of Newcastle Disease and Infectious Bronchitis. Cornell Vet., *40*, (1950): 39–48.

[26] Fabricant, J.: Studies on the Diagnosis of Newcastle Disease and Infectious Bronchitis of Fowls. IV. The Use of the Serum Neutralization Test in the Diagnosis of Infectious Bronchitis. Cornell Vet., *41*, (1951): 68–80.

[27] Fabricant, J.: A Modified Chick Embryo Inoculation Technique for the Isolation of Viruses from the Respiratory Tract of Chickens. Proc. Northeast. Conf. Lab. Workers Pullorum Dis. Control, Univ. Delaware, Newark, 1956.

[28] Fabricant, J., and Levine, P. P.: The Persistence of Infectious Bronchitis Virus in Eggs and Tracheal Exudates of Infected Chickens. Cornell Vet., *41*, (1951): 240–246.

[29] Fahey, J. E.: Tissue Culture Studies of the Avian Viruses. Proc. Northeast. Conf. Lab. Workers Pullorum Dis. Control, Univ. Delaware, Newark, 1956.

[30] Green, M., Stahmann, M. A., and Rasmussen, A. F., Jr.: Protection of Embryonated Eggs Infected with Infectious Bronchitis or Newcastle Disease Virus by Polypeptides. Proc. Soc. Exptl. Biol. and Med., *83*, (1953): 641–643.

[31] Groupé, V.: Demonstration of an Interference Phenomenon Associated with Infectious Bronchitis Virus (IBV) of Chickens. J. Bact., *58*, (1949): 23–32.

[32] Groupé, V., and Pugh, L.: Interference Between Influenza Virus and Infectious Bronchitis Virus of Chickens. J. Bact., *63*, (1952): 295–296.

[33] Groupé, V., Pugh, L. H., Weiss, D., and Kochi, M.: Observations on the Antiviral Activity of Viscosin. Proc. Soc. Exptl. Biol. and Med., *78*, (1951): 354–358.

[34] Hanson, L. E.: Separation of Newcastle Disease and Infectious Bronchitis Viruses in Mixed Infections. Poult. Sci., *33*, (1954): 223–230.

[35] Hanson, L. E., White, F. H., and Alberts, J. O.: Interference Between Newcastle Disease and Infectious Bronchitis Viruses. Am. J. Vet. Res., *17*, (1956): 294–298.

[36] Henle, W.: Interference Phenomena Between Animal Viruses: A Review. J. Immunol., *64*, (1950): 203–236.

[37] Hipolito, O.: Spectrophotometric and Serum Neutralization Studies of Sera from Chickens Exposed to Infectious Bronchitis Virus. Ph.D. thesis, Michigan State Univ., 1955.

[38] Hitchner, S. B., and White, P. G.: Growth-Curve Studies of Chick Embryo-Propagated Infectious Bronchitis Virus. Poult. Sci., *34*, (1955): 590–594.

[39] Hitchner, S. B., White, P. G., and Lozano, E. A.: Screening Tests on Modified Strains of Infectious Bronchitis Virus. American Scientific Laboratories, Inc., Madison, Wis., March, 1955.

[40] Hofstad, M. S.: A Study of Infectious Bronchitis in Chickens. I. The Pathology of Infectious Bronchitis. Cornell Vet., *35*, (1945): 22–31.

[41] Hofstad, M. S.: A Study of Infectious Bronchitis in Chickens. II. Observations on the Carrier Status of Chickens Recovered from Infectious Bronchitis. Cornell Vet., *35*, (1945): 32–35.

[42] Hofstad, M. S.: A Study of Infectious Bronchitis in Chickens. III. Attempts to Utilize the Chicken Red Cell Agglutination Test as a Diagnostic Aid in Infectious Bronchitis. Cornell Vet., *35*, (1945): 60–61.

[43] Hofstad, M. S.: A Study of Infectious Bronchitis in Chickens. IV. Further Observations on the Carrier Status of Chickens Recovered from Infectious Bronchitis. Cornell Vet., *37*, (1947): 29–34.

[44] Hofstad, M. S.: Infectious Bronchitis, in "Diseases of Poultry." H. E. Biester and L. H. Schwarte (ed.) 3rd ed. Iowa State College Press, Ames (1952): 509–519.

[45] Hofstad, M. S.: Stability of Avian Infectious Bronchitis Virus at 56 C. Cornell Vet., *46*, (1956): 122–128.

[46] Hofstad, M. S., and Kenzy, S. G.: Susceptibility of Chicks Hatched from Recovered Hens to Infectious Bronchitis. Cornell Vet., *40*, (1950): 87–89.

[47] Jungherr, E., Chomiak, T. W., and Luginbuhl., R. E.: Dosage Response of Tracheal Mucosa to Infectious Bronchitis Virus. Proc. Northeast. Conf. Lab. Workers Pullorum Dis. Control, Univ. Delaware, Newark, 1956.

[48] Jungherr, E. L., Luginbuhl, R. E., and Jacobs, R. E.: Pathology and Serology of Air Sac Infection. Proc. Book, AVMA (1953): 303–312.

[49] Jungherr, E. L., and Terrell, N. L.: Naturally Acquired Passive Immunity to Infectious Bronchitis in Chicks. Am. J. Vet. Res., *9*, (1948): 201–205.

[50] Levine, P. P.: The Distribution of P^{32} and S^{35} in Bronchitis Infected Chick Embryos. Proc. Northeast. Conf. Lab. Workers Pullorum Dis. Control, Univ. Delaware, Newark, 1956.

[51] Levine, P. P., and Hofstad, M. S.: Attempts to Control Air-Borne Infectious Bronchitis and Newcastle Disease of Fowls with Sterilamps. Cornell Vet., *37*, (1947): 204–211.

[52] Loomis, L. N., Cunningham, C. H., Gray, M. L., and Thorp, F., Jr.: Pathology of the Chicken Embryo Infected with Infectious Bronchitis Virus. Am. J. Vet. Res., *11*, (1950): 245–251.

[53] Luginbuhl, R. E., and Jungherr, E. L.: Simultaneous Titration of Newcastle Disease and Infectious Bronchitis Viruses in Embryonating Eggs. Poult. Sci., *32*, (1953): 911–912.

[54] Mathews, J., and Hofstad, M. S.: The Inactivation of Certain Animal Viruses by Ethylene Oxide (Carboxide). Cornell Vet., *43*, (1953): 452–461.

[55] Omeara, D. C.: Serum Absorption Studies of Infectious Bronchitis Virus. Proc. Northeast. Conf. Lab. Workers Pullorum Dis. Control, Univ. Delaware, Newark, 1956.

[56] Packer, R. A.: The Filterable Viruses and Bacteriophage, in "Veterinary Bacteriology and Virology." I. A. Merchant (ed.) 4th ed. Iowa State College Press, Ames, 1950.

[57] Page, C. A.: Antibody Response of Chickens to Infectious Bronchitis Virus. M.S. thesis, Michigan State Univ., 1950.

[58] Page, C. A.: A Study of the Serum Neutralization Test for Infectious Bronchitis of Chickens. Ph.D. thesis, Michigan State Univ., 1954.

[59] Reagan, R. L., and Brueckner, A. L.: Electron Microscope Studies of Four Strains of Infectious Bronchitis Virus. Am. J. Vet. Res., *13*, (1952): 417–418.

[60] Reagan, R. L., and Brueckner, A. L.: Response of the Swiss Albino Mouse to Infectious Bronchitis Virus. Poult. Sci., *32*, (1953): 1085–1086.

[61] Reagan, R. L., Brueckner, A. L., and Delaplane, J. P.: Morphological Observations by Electron Microscopy of the Viruses of Infectious Bronchitis of Chickens and the Chronic Respiratory Disease of Turkeys. Cornell Vet., *40*, (1950): 384–386.

[62] Reagan, R. L., Hauser, J. E., Lillie, M. G., and Craige, A. H., Jr.: Electron Micrograph of the Virus of Infectious Bronchitis of Chickens. Cornell Vet., *38*, (1948): 190–191.

[63] Reagan, R. L., Porter, J. R., Geumlek, M., and Brueckner, A. L.: Response of the Cave Bat to the Wachtel Strain of Infectious Bronchitis Virus. Southeast. Vet. (to be published). Cited from Reagan *et al.*, Poult. Sci., *34*, (1955): 1173–1174.

[64] Reagan, R. L., Yancey, F., and Brueckner, A. L.: Response of Young Rabbits to Infectious Bronchitis Virus. Poult. Sci., *34*, (1955): 1173–1174.

[65] Reed, L. J., and Muench, H.: A Simple Method for Estimating Fifty Per Cent End Points. Am. J. Hyg., *27*, (1938): 493–497.

[66] Shipkowitz, N. L.: Studies on the Adaptation of Infectious Bronchitis Virus to Mice. Ph.D. thesis, Michigan State Univ., 1952.

[67] Van Roekel, H.: Respiratory Diseases of Poultry, in "Advances in Veterinary Science." C. A. Brandly and E. L. Jungherr (ed.), Academic Press Inc., New York, 1955.

[68] Van Roekel, H., Bullis, K. L., Clarke, M. K., Olesiuk, O. M., and Sperling, F. G.: Infectious Bronchitis. Univ. Massachusetts, Agric. Exper. Sta. Bull. 460, 1950.

[69] Van Roekel, H., Clarke, M. K., Bullis, K. L., Olesiuk, O. M., and Sperling, F. G.: Infectious Bronchitis. Am. J. Vet. Res., *12*, (1951): 140–146.

[70] van Rooyen, C. E.: A Revision of Holmes's Classification of Animal Viruses, Suborder III (Zoophagineae), Canad. J. Micros., *1*, (1954): 227–284.

Pathogenic and Nonpathogenic Pleuropneumonia-like Organisms in Infectious Sinusitis of Turkeys

H. E. ADLER, D.V.M., Ph.D., and R. YAMAMOTO, M.A.

Davis, California

THIS PAPER describes the isolation and properties of two strains of pleuropneumonia-like organisms (PPLO) from sinus exudate of a turkey during attempted virus isolation. One strain was streptomycin resistant and pathogenic and did not readily form colonies on serum agar. The other strain was streptomycin susceptible and could be propagated easily on PPLO agar.

MATERIALS

Two turkeys exhibiting marked respiratory distress were submitted to the laboratory of the School of Veterinary Medicine for diagnosis. The undiluted serums of these birds contained agglutinins for PPLO.[1] At necropsy, both birds showed a clear albuminous exudate in the infraorbital sinuses, mucopurulent tracheitis, and aerosaceulitis. Histological examination of an affected sinus revealed a lymphofollicular reaction characteristic of that present in chronic respiratory disease.[2]

PROCEDURES AND RESULTS

Sinus exudate was aspirated, pooled, and seeded on selective PPLO broth and agar,[2] thiol medium, blood agar, and Sabouraud's medium. All cultures were negative for bacteria but colonies of PPLO were observed on the serum agar. An antigen prepared from the PPLO was not agglutinated by hyperimmune serum produced from a known pathogenic strain of PPLO of turkey origin. A third culture passage of the PPLO on agar was inoculated into the infraorbital sinuses of 4 poults and 6 chickens. They were observed for 34 days. No symptoms or lesions appeared and the serums of all birds were negative for PPLO agglutinins.[1]

Concurrently with the PPLO studies, an attempt was made to isolate a virus or viruses from the turkey sinus exudate. One-tenth milliliter of a 10 per cent dilution of exudate containing 2.5 mg. of streptomycin and 1,000 units of penicillin was inoculated via the yolk sac, allantoic sac, and on the chorioallantoic membrane, respectively, into fertile chicken eggs previously incubated for six to eight days. Lesions produced on the chorioallantoic membrane (fig. 1) were similar to the pocks described by Delaplane for the chronic respiratory disease agent [3] and for a virus [4] associated with this condition. A consistent embryo mortality pattern occurred only in embryos inoculated by the yolk sac route. Serums containing antibodies for infectious bronchitis, Newcastle disease, laryngotracheitis, and fowl pox failed to alter either the embryo lesions or mortality rate as determined by routine neutralization procedures. Mice that were injected intraperitoneally and intranasally with yolk from affected chicken embryos failed to show signs of disease. Three blind passages of ground lung in mice by the same routes of inoculation were negative.

Ten cockerels and 2 poults whose serums were negative for PPLO agglutinins were inoculated with yolk from the ninth serial passage in eggs. Culture of the inoculum for each passage did not reveal PPLO. All birds had respiratory râles within four to 17 days. The chickens showed signs much later than did the turkeys. At necropsy, 22 days after inoculation, PPLO agglutinins were present in the serums of the infected birds. Pathological changes typical of the disease were seen. In view of the findings, the presence of PPLO in the inoculum was suspected. The original work of Nelson [7] pointed out the advantage of tissue culture for the propagation of PPLO. Accordingly, a sample of the same yolk used to infect the chickens and turkeys was seeded into several tubes of tissue culture medium prepared by the method of Nelson; however, Simms-Sander's balanced salt solution [8] was used in place of Tyrode's fluid.

Ten tubes of medium and minced cells served as controls to indicate whether PPLO had been introduced from the minced cell suspension. Acid was produced in the tubes inoculated with yolk sac material

From the Department of Poultry Pathology, School of Veterinary Medicine, University of California, Davis.

The research was supported in part by a grant-in-aid to the University of California by the American Cyanamid Co., Lederle Laboratories Division, Pearl River, N.Y.

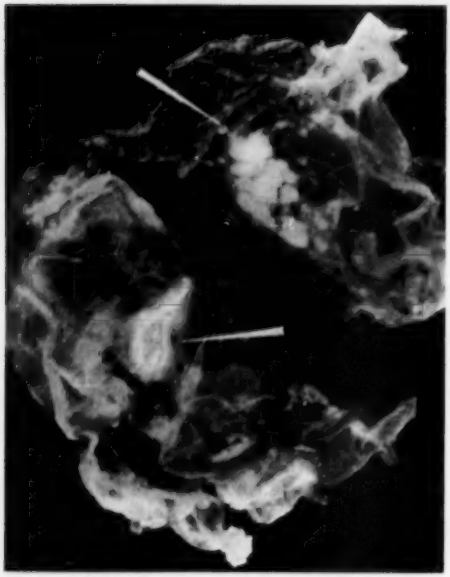


Fig. 1—At lower left, a plaque (arrow), on the chorioallantoic membrane of a 7-day-old embryo, produced by the PPLO. Upper right are pocks (arrow) on the chorioallantoic membrane caused by laryngotracheitis virus infection.

within six days. The entire tube of medium changed from red to canary yellow. Smears fixed in Bouin's and stained with Giemsa solution revealed large numbers of blue- and purple-staining coccoid bodies approximately 0.25 to 0.5 μ in size. The presence of coccoid bodies, the utilization of glucose with the production of acid,[5] and the absence of bacteria on serum agar subculture suggested the presence of PPLO, even though the organisms failed to colonize on serum-enriched agar to fulfill the requirements for PPLO identification established by Sabin.[8]

To determine whether PPLO was the cause of the infectious sinusitis and the embryo lesions, exudate from the poults infected with the ninth passage yolk material was subcultured in cell-free PPLO broth six successive times using 1 drop to seed 2.0 ml. of broth. One-tenth milliliter of the latest culture was inoculated into the infraorbital sinuses of 7 poults. Within 96 hours, 4 inoculated poults had sinusitis. Exudate was harvested from the affected sinuses and pooled for further studies. Cultures were made for PPLO in broth and tissue culture medium. Acid was formed in the tissue culture within three days and a large number of coccoid bodies were seen when stained. A few PPLO colonies were observed[1] on agar subculture from broth after ten days' incubation.

In addition, exudate from the above poults was placed on the chorioallantoic membrane of 7-day-old embryos. Discrete lesions were observed on the membranes after five days' incubation. Finally, an attempt was made to determine the antibiotic sensitivity of this strain of PPLO. Significant protection of embryos was provided by 1.0 mg. per embryo of erythromycin, oxytetracycline, chlortetracycline, and tetracycline. Streptomycin at this level was not effective in prolonging the life of the embryos.

CONCLUSIONS

In view of these findings, it was concluded that two strains of pleuropneumonia-like organisms (PPLO) were present in the original turkey sinus exudate. One strain was readily propagated on artificial medium and was not pathogenic for turkeys and chickens. The second strain of PPLO did not readily colonize on serum-enriched agar and was capable of producing disease. The early observations suggesting virus etiology were not borne out by subsequent cultures and animal infectivity studies.

References

[1] Adler, H. E., and Yamamoto, R.: Preparation of a New Pleuropneumonia-like Organism Antigen for the Diagnosis of Chronic Respiratory Disease by the Agglutination Test. Am. J. Vet. Res., *17*, (1956): 290–293.

[2] Adler, H. E., Yamamoto, R., and Bankowski, R. A.: A Preliminary Report of Efficiency of Various Mediums for Isolation of Pleuropneumonia-like Organisms from Exudate of Birds with Chronic Respiratory Disease. Am. J. Vet. Res., *15*, (1954): 463–465.

[3] Delaplane, J. P.: Some Recent Observations of Lesions in Chick Embryos Induced by the Virus of Chronic Respiratory Disease of Chickens. Cornell Vet., *38*, (1948): 192–195.

[4] Fahey, J. E.: A Virus in Chronic Respiratory Disease of Turkeys. Nature, *177*, (1956): 90.

[5] Grumbles, L. E., Phillips, E., Boney, W. S., and Delaplane, J. P.: Cultural and Biochemical Characteristics of the Agent Causing Infectious Sinusitis of Turkeys and Chronic Respiratory Disease of Chickens. Southwest. Vet., *6*, (1953): 166–168.

[6] Jungherr, E.: The Pathology of Experimental Sinusitis of Turkeys. Am. J. Vet. Res., *10*, (1949): 372–383.

[7] Nelson, J. B.: Studies on an Uncomplicated Coryza of the Domestic Fowl. VII. Cultivation of the Coccobacilliform Bodies in Fertile Eggs and in Tissue Culture. J. Exptl. Med., *64*, (1936): 749–758.

[8] Sabin, A. G.: The Filterable Microorganism of the Pleuropneumonia Group. Bact. Rev., *5*, (1941): 1–67.

[9] Sanders, M., Kiem, I., and Lagunoff, D.: Cultivation of Viruses. Arch. Path., *56*, (1953): 148–225.

Observations on a Chicken Embryo Lethal Orphan (CELO) Virus

VANCE J. YATES, D.V.M., and DOROTHY E. FRY, B.A.

Kingston, Rhode Island

DURING the past decade, embryonating eggs have been used extensively in research and in the production of vaccines. From time to time, investigators have found that these embryonating eggs contained contaminating microorganisms. Some of the organisms are known to be the causative agents of diseases of chickens. A review of this subject was written by Cottral in 1952.[1] He listed five viral and four bacterial diseases of birds in which evidence suggested that egg transmission occasionally took place. Since that time, Van Roekel and Olesiuk [2] reported that at least one more agent causing a disease in chickens should be added to this list.

This paper gives some observations on the isolation and characterization of the causative virus of another infection of chickens that appears to be occasionally transmitted by the ovarian route. The agent under consideration has been named CELO, since it produces death when introduced into embryonating chicken eggs and its virulence can only be consistently detected by this means.

The Isolation of the Agent.—In August, 1952, a serum sample was obtained by the University of Rhode Island laboratory from a cow which showed enlargement of the superficial lymph nodes. The serum was treated with streptomycin (33 mg./ml.) to control bacterial contaminants and inoculated into the allantoic sac of embryonating chicken eggs. A set of 6, 7-day-old embryos was used. One embryo died on the sixth day of incubation following inoculation and the allantoamnionic (a.a.) fluid was harvested. The a.a. fluid was used to inoculate a second set of 6, 7-day-old embryos. Ten days after inoculation, 2 embryos that appeared dwarfed were selected and their a.a. fluids harvested. In the third passage, 7-day-old embryos were again used for inoculation and all died by the seventh day following injection. For the fourth and subsequent passages, 11-, 12-, or 13-day-old embryos were used. With but a few exceptions, all inoculated embryos were dwarfed and succumbed to the agent on the third, fourth, or fifth day after inoculation.

A second serum sample was obtained from the same animal two months after the first. This sample was used to inoculate both 7- and 10-day-old embryonating eggs, but three serial passages failed to indicate the presence of any infective agent.

This CELO agent strain was designated Panciera, and some further preliminary studies were made before the pressure of other research caused temporary discontinuance of the work.

The Isolation of Other Strains of CELO Virus.—From May through July, 1953, an agent that caused consistent deaths in embryonating eggs was isolated on three occasions. The materials used for these isolations were of varied origin; a brain suspension of a calf with a nervous disorder (Phelp's strain), a suspension of the respiratory tract of a starling, and tracheal exudate of a cockerel showing respiratory difficulties.

When it was realized that the lesions produced in embryonating eggs were similar in each case and as the embryo death and lesion pattern was unfamiliar, it was decided to investigate the source of the eggs used in the diagnostic laboratory.

Single Comb White Leghorn hens producing the eggs for the laboratory were progeny of the University flock and had been apparently healthy from hatching. They had been exposed to the laboratory strain of infectious bronchitis virus (IBV) and vaccinated for Newcastle disease (ND) via the wing-web during the growing period. As an initial step in this study, blood

The authors are members of the Department of Animal Pathology, University of Rhode Island, Kingston.

Part of the studies reported here were carried out at the University of Wisconsin.

Contribution 897 of the Rhode Island Agricultural Experimental Station.

Presented before the Combined Sections on Poultry and Research, Ninety-Third Annual Meeting, American Veterinary Medical Association, San Antonio, Texas, Oct. 15–18, 1956.

samples were taken from all the hens and each serum sample was mixed with at least 10^2 embryo lethal doses$_{50}$ (e.l.d.) of one strain (Phelp's) of the CELO agent. After incubation, the mixture was used to inoculate 11-day-old embryonating eggs. Neutralization of the agent occurred with over half of the serums tested. The birds were divided on the basis of this test into two lots and mated. One pen contained 20 hens that were carrying antibodies against the virus and the other pen contained 15 birds which did not have sufficient humoral antibodies to neutralize the agent used. The birds were trapnested and all eggs laid were identified and observed daily from the seventh through the twentieth day of incubation. The a.a. fluid of embryos dying or showing retarded growth was harvested and saved for isolation trials. The birds in both groups appeared normal and their production and hatchability were good. All isolation attempts using the a.a. fluid harvested from the dead or abnormal embryos were unsuccessful. A second study of the serums of birds in the two pens five months after separation revealed that all but 1 bird then had significant neutralizing antibodies against the Phelp's strain.

The Characteristics of CELO Virus.—The original isolation of two of the seven strains studied was made when 7-day-old embryos were inoculated with streptomycin-treated suspensions of animal tissues or exudates. Embryos that were 10, 11, or 12 days old were used in isolating the other strains and in each case streptomycin was used to control bacterial contaminants. All inoculations were made into the allantoic cavity and, after four to six passages, all strains studied produced consistent and similar lesions and death patterns. All infected embryos were noticeably dwarfed and succumbed to the agent in two to five days following inoculation. Some of the embryos resembled those infected with IBV in size, but they were not tightly curled nor did the amnionic membrane adhere as closely to the embryos. Histological studies of a limited number of infected embryos destroyed the third day following inoculation failed to show definite lesions.

The CELO virus caused death or dwarfing or both when it was inoculated into embryonating chicken eggs from the sixth through the thirteenth day of incubation. When it was used in 6-day-old embryos, inoculation into the yolk sac resulted in a more uniform death time. In embryos 8 through 12 days of age, the agent produced a consistent mortality and lesion pattern. Considering the titer of the agent in embryos of different ages, it appeared that 10- and 11-day-old embryos were the most desirable for its propagation. In embryos of this age, inoculated via the allantoic route, the e.l.d.$_{50}$ titer of a.a. fluid ranged from 10^9 to 10^{11}.

The agent also produced deaths but no distinct gross lesions when it was inoculated onto the chorioallantoic membrane.

Two strains (Phelp's and Panciera) were filtered through the Berkefeld (W) candle. The filtered material produced typical lesions in the chicken embryo. The Phelp's strain was retained by the Seitz EK filter. Other strains of the agent were not tested.

Amounts of 1.0 ml. of undiluted a.a. fluid were sealed in glass tubes and put in a 56 C. water bath. At 60 and 90 minutes, the agent still produced death when inoculated into 11-day-old embryos, but at 105 minutes the virus was apparently inactive.

Animal Inoculations.—Previous work with chickens indicated that they were not susceptible to the CELO agent by intravenous, intraperitoneal, intramuscular, and intratracheal routes, or by inoculation into the thoracic air sacs. This work did show, however, that a few birds exhibited nervous signs and finally succumbed to intracerebral inoculation.[3,4] It was also reported that turkeys were not susceptible by intramuscular, intracerebral, and conjunctival plus infraorbital sinus inoculation with the CELO agent.[4] Quail were also found to be refractory to conjunctival and intramuscular inoculations.[4] Rabbits were not susceptible to intravenous inoculations, but they responded with a high level of neutralizing antibodies specific for the virus.[3]

Suckling mice inoculated intracerebrally with 0.03 ml. of a.a. fluid failed to show any signs of disease.

Relationship of CELO Virus to Other Animal Diseases, Especially Those of Chickens.—Rabbit or chicken antiserum against the viruses of ND, IB, laryngotracheitis, fowlpox, and mumps did not neutralize the agent. Anti-CELO serum produced in rabbits did not significantly affect the titer of IBV or NDV in chicken embryos.

The titer of avian encephalomyelitis (AE) virus in young chickens was not altered by adding anti-CELO serum to the inoculum, nor was the titer affected when CELO-immune chicks were inoculated. In one trial, the two agents were mixed and inoculated intracerebrally into young chickens. The signs and deaths from AE were retarded.

The CELO virus appeared to be related to the viral agent (quail bronchitis) isolated by Olson in 1949.[5] Antiserum against the CELO virus produced in rabbits neutralized 10^7 e.l.d.$_{50}$ of the Olson virus. The lesion and death pattern in 10- and 11-day-old embryos appeared to be similar for both agents.

Occurrence.—Since the various strains studied did not produce clinical signs in chickens by most routes of inoculation, the occurrence of present or past infections was studied by the use of serum-neutralization tests in embryonating eggs. In the usual test, heat-inactivated serum was mixed with at least 10^2 e.l.d.$_{50}$ of one strain of the CELO agent. After an incubation period of approximately 30 minutes, the mixture was used to inoculate 10- or 11-day-old chicken embryos via the allantoic sac. The survival of over half of the inoculated embryos was considered as indicating significant humoral antibodies against the agent.

Using this procedure, the birds in 42 of the 43 pens in the 1954–1955 Rhode Island egg-laying contest were tested.[4] Birds submitted to the contest are from many of the important poultry-producing areas of the United States and it was felt that a study of these birds at the time of submission would give some valuable information as to the occurrence of this infection.

Birds in 37 of the 42 pens tested showed evidence of neutralization.

Serum samples from a few randomly selected adult birds from the U.S. Regional Poultry Research Laboratory at East Lansing, Mich., were also tested.[4] There was significant neutralization in 37 of the 43 samples from the four nonisolated lines and in three of the 11 samples from the four pens of isolated line 15. The latter three positive samples were all from birds in one pen. A few serum samples collected at the same laboratory three years previously (1952) were also tested but none of these neutralized the test strain.

Individual serum samples collected from 4 or 5 birds, in each of 12 commercial poultry flocks of Wisconsin, were tested. In 11 of the 12 flocks, a majority of the serum samples showed evidence of a present or past infection. The occurrence of the disease was also studied by using pooled yolk samples in place of serum for the neutralization test. This procedure was used to test five hatchery supply flocks and all but one showed evidence of contact with the CELO virus.

Discussion

The isolation of the CELO virus from embryonating eggs, inoculated with tissue or exudates or both, of varied origin indicated that it was likely an endogenous virus of the egg. The finding of significant neutralizing antibodies in many of the birds in the University supply flock supported the hypothesis. As the CELO virus has only been isolated occasionally, it seems logical to assume that the agent was only present in the eggs from these carrier birds during a limited period of time, perhaps the first week after contact. When the normal physiology of the infected embryonating eggs was altered by inoculation or by some other means, the agent was revealed. All strains studied were isolated when antibiotics were a part of the inoculum, suggesting that the antibiotics might have been a factor in disturbing the developing embryo.

Limited investigation was done to characterize the CELO agent. After the agent became adapted to chicken embryos, it was easily propagated, as embryos of various ages could be used and its pathological effects were consistent. The filtration studies indicated only that the agent was probably a virus. The agent was resistant to inactivation by heating, also to the action of streptomycin.

The CELO virus appeared to be serologically distinct from the other widely studied viruses of chickens. It has not been determined, however, if this normally inapparent agent is a contributing factor in any disease of chickens. The inoculation of young chickens with a mixture of AE and CELO virus retarded the development of the nervous manifestations of AE. This observation seems to indicate that these two agents may invade the same tissue cells of the body. As the incubation time

following intracerebral inoculation of the CELO agent into young chicks usually exceeds two weeks, the first signs of a dual infection (CELO plus AE) should be those of AE. This appeared to be the case.

The results of the neutralization tests indicated that this agent was widespread. This situation has likely resulted from more than one method of transmission. Besides ovarian transfer, the agent may be spread by aerosol or some other means. From limited observations, it appeared that the agent is not highly contagious.

If as suggested, the virus is sometimes found in the fertile eggs of apparently normal chickens, then the possible presence of this agent must be taken into account in vaccine production and in other procedures employing embryonating eggs. When the agent becomes adapted to chicken embryos it frequently produces dwarfing similar to that observed in those inoculated with IBV. This may be a confusing factor in the diagnosis of IB and possibly other virus diseases.

SUMMARY

The isolation of seven strains of a viral agent (CELO virus) that produced consistent embryo lesions and death patterns is reported. All isolations were made from chicken embryos that were inoculated with varied tissues or exudates treated with streptomycin to control bacterial contaminants.

Serologically, the strains of the CELO virus studied were not related to the causative agents of infectious bronchitis, Newcastle disease, laryngotracheitis, fowlpox, and avian encephalomyelitis.

Neutralizing antibodies against the CELO virus were found in the serum of chickens from some of the major poultry-producing areas of the United States.

References

[1] Cottral, G. E.: Endogenous Viruses in the Egg. Ann. New York Acad. Sci., *55*, (1952): 221–234.

[2] Van Roekel, H., and Olesiuk, O. M.: The Etiology of Chronic Respiratory Disease. Proc. Book, AVMA (1953): 289–301.

[3] Yates, V. J., Fry, D. E., and Wasserman, B.: A Preliminary Report on an Apparently New Virus Disease of Chickens. Proc. Ann. Conf. Lab. Workers in Pullorum Dis. Control, June, 1954.

[4] Yates, V. J., and Fry, D. E.: Further Studies of the Inapparent Virus Disease of Chickens. Proc. Ann. Conf. Lab. Workers in Pullorum Dis. Control, June, 1955.

[5] Olson, N. O.: A Respiratory Disease (Bronchitis) of Quail Caused by a Virus. Proc. U. S. Livestock San. A. (1950): 171–174.

A Comparison of the Protection-Inducing Factors in Chickens and Mice of a Type 1 Strain of *Pasteurella Multocida*

KATHERINE E. YAW, Ph.D., and J. C. KAKAVAS, Ph.D.

Newark, Delaware

THE TYPE-SPECIFIC capsular polysaccharide of *Pasteurella multocida* has been reported to give protection to mice challenged with virulent microorganisms of a homologous type but there was no protection after challenge with bacteria of other serological types.[3]

Smooth or fluorescent colonial variants of strains of three different serological types of *Past. multocida*, though equally virulent for mice, vary markedly in their virulence for chickens.[9] Since different components of cells of *Pasteurella pestis* are responsible for immunization in different hosts,[5] experiments were planned with a type 1 strain of *Past. multocida* (Little and Lyon classification [6]) which is highly virulent for both chickens and mice to determine whether the protection-inducing factors of this strain are the same for both hosts.

MATERIALS AND METHODS

Cultures Used and Their Maintenance.—A type 1 strain, 9137, of *Past. multocida* was used in this investigation, since it is highly virulent for mice and chickens when cells of the encapsulated, fluorescent (F) dissociant [4] are injected. The cells of the unencapsulated or rough, blue (B) variant [4] are of low virulence for both hosts. A type 3 strain, 6535, which is avirulent for chickens but virulent for mice was also used in this study.

The methods and mediums used in isolating, maintaining, and growing the desired colonial variants, as well as the cultural and biochemical characteristics of these strains, have been reported previously.[9]

Experimental Animals.—The N.I.H. strain of mice (18 to 20 Gm.) was used and immunizing and challenge injections were given intraperitoneally. Rhode Island Red chickens were 2 weeks old when immunizing injections, given intramuscularly in the thigh, were started. Challenge doses were given by the same route when the birds were 4 weeks old. Both hosts were kept seven days after challenge and all deaths reported occurred during that period.

Preparation of Immunizing Antigens.—Crude preparation of capsular polysaccharide from the F dissociant of strain 9137 was prepared in part by the method of Carter.[2] The bacteria were grown for 18 to 20 hours at 37 C. on the chosen medium in 40 large (22 by 200 mm.) tubes. The growth was removed from the agar surfaces with physiological saline, and the cells were washed three times with the same solution and resuspended in 10.0 ml. of saline solution. The bacterial suspension was then heated in a water bath at 56 C. for one hour to partially remove the capsular material and to kill the microorganisms. Following the heat treatment, the suspension was centrifuged, the supernatant containing the capsular polysaccharide was saved for animal injection, and the mass of bacterial cells was saved for further use. Many 10.0-ml. portions thus prepared were combined and designated as capsular antigens. Additional lots of capsular material, prepared in the same way, were Seitz-filtered before injection into animals to remove any bacterial cells left in the supernatant after centrifugation.

The F cells of strain 9137 remaining, after the removal of the supernatant which contained the capsular polysaccharide, did not agglutinate in rabbit antiserum induced by the injection of formalin-killed 9137 B cells. However, after repeated washing in physiological saline, samples of the bacterial cells did agglutinate in 9137 B antiserum to full titer. This agglutination of the cells indicated that all capsular material had been removed and that the somatic antigen(s) were exposed. These decapsulated washed cells were diluted in 0.2 per cent physiological saline solution, adjusted to a MacFarland nephelometer standard 3 and designated as vaccine 1.

The B colonial variant of strain 9137 was grown for 18 to 20 hours at 37 C. in the chosen broth medium, centrifuged, and the living cells resuspended in sterile, distilled water, adjusted to the same turbidity as vaccine 1 and stored in an icebox. Autolysis of the bacterial cells in this menstruum lowered the count of viable bacteria to approximately 5×10^4 at the time of injection. This number of living cells of the B variant was not virulent for either host. This living suspension of cells was designated as vaccine 2.

A killed vaccine prepared from cells of the B variant was designated as vaccine 3. The bacteria were grown for 18 to 20 hours at 37 C. on the chosen medium, removed from the agar surface into physiological saline, washed once, resuspended in 0.2 per cent formalized physiological

From the Department of Biological Sciences, University of Delaware, Newark.

This research was supported by a grant from the University of Delaware Research Foundation.

saline, and adjusted to the same turbidity as vaccine 1.

The vaccine made from cells of the F variant of the type 3 strain, 6535, was prepared in the same way.

Preparation of Challenge Inoculum.—Virulent bacteria isolated from the F colonial variant of strain 9137 were always used as challenge inoculums. The methods for its preparation have been published.[9] The number of bacteria in the 0.2 ml. of the 10^{-8} dilution was less than ten and there was a tenfold increase in numbers of bacteria injected with each lower dilution of inoculum.

RESULTS

The results of experiment 1 (table 1) indicate that one injection of the unencapsulated living B colonial variant of this type 1 strain and three injections of the capsular polysaccharide solution protected chickens that received challenge doses of 10^2 and 10^4 times greater than that required to kill 80 and 90 per cent, respectively, of the control animals. (Unreported preliminary experiments in our laboratory showed that the cells of unencapsulated variant did effectively immunize chickens and, since it was assumed that the capsular material would not protect, more injections of the capsular material were given to the chickens.) In mice, the capsular antigen protected against approximately 10^3 times the number of bacteria that killed 90 per cent of the control animals. However, the somatic antigens of the vaccine gave little protection to the host even with as small a challenge dose as less than ten virulent bacteria.

In these experiments, necropsies were performed on all immunized animals which died and approximately 50 per cent of the controls, and the livers were cultured. Pure cultures of *Past. multocida* were isolated from all livers unless otherwise stated.

In experiment 2 (table 1), changes were made in the preparation of the immunizing antigens to strengthen the proof that the somatic or intracellular antigens of the unencapsulated cells of the B variant, as well as the capsular antigen, were able to protect chickens while only the capsular antigen effectively immunized mice. A for-

TABLE 1—Protection in Chickens and Mice After Immunization with Capsular and Somatic Antigens of Type 1 Strain of Pasteurella Multocida

Host	Immunizing antigens	No. of injections*	Amount/injection	Dilution of challenge injected	Survivors/injected	Percentage survival of animals
		EXPERIMENT 1				
Chicken	Somatic vaccine 2	1	0.2 ml.	10^{-2}	9/10	90
	Somatic vaccine 2	1	0.2 ml.	10^{-3}	10/10	100
	Capsular	3	0.5 ml.	10^{-2}	10/10	100
	Capsular	3	0.5 ml.	10^{-3}	10/10	100
	Controls	—	—	10^{-2}	0/10	0
	Controls	—	—	10^{-3}	2/10	20
	Controls	—	—	10^{-7}	1/10	10
Mouse	Somatic vaccine 2	2	0.2 ml.	10^{-7}	0/10	0
	Somatic vaccine 2	2	0.2 ml.	10^{-8}	3/10	30
	Capsular	2	0.5 ml.	10^{-3}	9/10	90
	Controls	—	—	10^{-7}	0/10	0
	Controls	—	—	10^{-6}	1/10	10
		EXPERIMENT 2				
Chicken	Somatic vaccine 1	3	0.2 ml.	10^{-2}	9/10	90
	Somatic vaccine 3	3	0.2 ml.	10^{-3}	9/10	90
	Capsular (Seitz-filtered)	3	0.5 ml.	10^{-3}	10/10	100
	Controls	—	—	10^{-3}	0/10	0
	Controls	—	—	10^{-7}	2/10	20
Mouse	Somatic vaccine 1	3	0.2 ml.	10^{-8}	4/10	40
	Somatic vaccine 3	3	0.2 ml.	10^{-8}	5/10	50
	Capsular (Seitz-filtered)	3	0.5 ml.	10^{-6}	9/10†	90
	Controls	—	—	10^{-7}	0/10	0
	Controls	—	—	10^{-8}	0/10	0
		EXPERIMENT 3				
Chicken	Somatic vaccine 3	1	0.2 ml.	10^{-4}	10/10	100
	Capsular-somatic type 3 vaccine	1	0.2 ml.	10^{-4}	0/10	0
	Controls	—	—	10^{-4}	0/10	0
	Controls	—	—	10^{-5}	1/10	10

* Interval between injections was four days; interval between first immunizing injection and challenge was two weeks.

† A Pseudomonas sp. isolated from mouse that died.

malin-killed suspension of cells of the B variant was used to eliminate the possibility that some metabolic product produced by the living bacteria in the infected host was an immunizing factor. The Seitz filtration of the capsular solution removed all bacterial cells that remained after centrifugation. Also, if cells of the F variant differ from cells of the B variant only in the possession of a capsule, then upon removing this capsule the antigen(s) present should be common to both cells, the F and its B dissociant. With vaccine 1, we attempted to evaluate this assumption. The number of immunizing injections and the amounts injected were the same for both hosts. A challenge dose which was 10^2 times greater than that which normally killed control animals was given the immunized chickens and mice. The results obtained in this experiment corroborate the results obtained in experiment 1. The bacteria with the exposed somatic antigens give protection only to chickens, while the solutions containing the capsular material give protection to both hosts.

Since the somatic antigen(s) of *Past. multocida* are reported by Carter [2] to be common to all the serological types of this species and since somatic antigen(s) protect chickens, a vaccine consisting of any heterologous type of this species should protect chickens. Positive results from such an experiment would be in contradiction to results published previously.[9] However, it is evident from experiment 3 that chickens vaccinated with type 3 strain, 6535 F, were not protected when challenged with a strong dose of virulent type 1 culture.

Discussion

In the experiments reported in this paper, the capsular and somatic antigens present in strain 9137 of the serological type 1 of *Past. multocida* were tested in two hosts, chickens and mice, for their ability to immunize them against the virulent dissociant of the same strain. The maximum amount of protection possible for either host by the injection of these antigens was not stressed. A crude soluble preparation from the cells was used for the capsular antigen while intact cells of the unencapsulated B variant were used as the source of somatic antigen(s). The results of these experiments, instead of clarifying the mechanism(s) of protection against this bacterium, add to the complexity of the problem; or else, they indicate that our understanding of the dissociation pattern and of differences in serological types is either incorrect or more incomplete than hitherto anticipated.

The type-specific capsular material of a type 2 strain of *Past. multocida* has been reported to protect mice [3] and the results of these experiments using a type 1 strain agree with the reported results. The same crude preparation of capsular polysaccharide also protects chickens. The somatic antigens of this strain of bacterium are not protective for mice but are protective for chickens. A difference in the protection-inducing factors in different hosts has been reported for *Past. pestis* [1] whose somatic antigens protect guinea pigs but not rats and mice.

The mechanism of an immune response by which both the capsular polysaccharide and the somatic antigen(s) of *Past. multocida* protect chickens defies precise interpretation. One possible explanation for such a mechanism might be that a common capsular antigen is present in varying amounts on both the encapsulated and the unencapsulated cells and that this common antigen is differentially more antigenic for chickens than mice. Carter [2] reported that strains which yield a large proportion of B variants do possess a small amount of capsular polysaccharide. While studying the mechanism of protection in chickens immunized with the unencapsulated B variant, there has been some evidence of increased phagocytosis of the encapsulated virulent cells of the F variant used in the challenge. This antigen-antibody reaction strongly suggests an antigen present on the cells of the B variant which is either the same polysaccharide or so closely similar chemically that cross reactions occur.

Carter [2] reports that *Past. multocida* has, in addition to the type-specific capsular material, a somatic antigen which is common to all members of this species. Priestley,[7] however, reports differences in somatic antigens between strains isolated from cattle and those isolated from fowl. A recent report by Doubly,[4] in which the colonial variants used in the production of rabbit antiserum with high agglutinin titer are not mentioned, suggests the possibility of different somatic antigens, since the variants with the type-specific capsule are almost inagglutinable. The results re-

ported in this paper suggest the presence of different somatic antigens (or possibly intracellular antigens) in strains of different serological types. There might be a common somatic antigen present in all members of the species and different additional somatic antigens on the different serological types.

The virulence for chickens and mice of the type 1 strain of *Past. multocida* used in this study has been reported.[9] It has been noted during the past year that while the virulence of this strain for mice has been constant, the virulence for chickens has decreased. It has not been possible to attribute this decrease in virulence to subclinical infection in the control animals and it has not been possible to restore the virulence by a single passage through chickens. Previously, it was possible to kill all chickens with less than 100 virulent bacteria in two to three days. Within the last year, the time required and the number of bacteria necessary to kill have both increased. It is possible that for this host, there is an additional virulence factor which is lost after prolonged cultivation on artificial medium.

SUMMARY

Crude preparations of capsular polysaccharide from a type 1 strain of *Pasteurella multocida* protect chickens and mice against challenge with the virulent bacteria of this strain. Somatic or intracellular antigens of the B variant, or both, protect chickens but do not protect mice when challenged with the virulent culture. Possible explanation of these phenomena are discussed.

It is suggested that the protective somatic antigen(s) of the various serological types may differ.

References

[1] Baker, E. E., Sommer, H., Foster, L. E., Meyer, E., and Meyer, K. F.: Antigenic Structure of *Pasteurella Pestis* and the Isolation of a Crystalline Antigen. Proc. Soc. Exptl. Biol. and Med., *64*, (1947): 139–141.

[2] Carter, G. R.: The Type Specific Capsular Antigen of *Pasteurella Multocida*. Canad. J. M. Sci., *30*, (1952): 48–53.

[3] Carter, G. R., and Annau, E.: Isolation of Capsular Polysaccharides from Colonial Variants of *Pasteurella Multocida*. Am. J. Vet. Res., *14*, (1953): 475–478.

[4] Doubly, J. A.: Diverse Antigenic Character of *Pasteurella Multocida*. Bact. Proc. (1956): 98.

[5] Hughes, T. P.: The Epidemiology of Fowl Cholera. II. Biological Properties of *P. Avicida*. J. Exptl. Med., *51*, (1930): 225–238.

[6] Little, P. A., and Lyon, B. M.: Demonstration of Serological Types Within the Nonhemolytic Pasteurella. Am. J. Vet. Res., *4*, (1943): 110–112.

[7] Priestley, F. W.: Some Properties of the Capsule of *Pasteurella Septica*. Brit. J. Exptl. Path., *17*, (1936): 374–378.

[8] Schütze, H.: Studies on *P. Pestis* Antigens: The Prophylactic Value of Envelope and Somatic Antigens. Brit. J. Exptl. Path., *13*, (1932): 293–298.

[9] Yaw, K. E., Briefman, L., and Kakavas, J. C.: A Comparison of Virulence for Mice and Chickens of Different Colonial Variants of the Three Serological Types of *Pasteurella Multocida*. Am. J. Vet. Res., *17*, (1956): 157–159.

Surgical Technique for the Exteriorization of the Ureters of the Chicken

J. M. DIXON, D.V.M., and W. S. WILKINSON, Ph.D.

Baton Rouge, Louisiana

METABOLISM STUDIES in chickens and other fowl have been handicapped due to the common excretory pathway for urine and fecal matter. Surgical techniques allowing separate collection of urine and feces have been reported, but these are not adaptable where larger numbers of birds are to be used in long-term experiments.

In 1886, a method of separate urine collection for a short time was reported.[1] It involved ligation of the rectum anterior to the cloaca of anesthetized geese. In 1903, a similar technique was described in which an artificial anus was created to prevent fecal contamination of the urine during longer periods of study.[2]

In 1940, an operation was developed for exteriorization of the ureters of hens in which the dorsal portion of the cloaca was elevated on the pygostyle.[3] A modification of this technique was used in 1955 to study the incorporation of the methyl group of methionine into urinary uric acid in 5-week-old chicks.[4]

In 1942, two surgical methods which allowed separate urine collection were investigated.[5] A method was developed for exteriorization of the ureters dorsal to the vent through a fistulous tract which proved to be superior to the creation of an artificial anus, particularly for long-term studies.

The surgical technique described herein is similar to the last one [5] except that the fistulous tract to the rectum has been eliminated. The operation is rapid and simple which allows large numbers of birds to be so modified and once healing has occurred, the bird may be used indefinitely.

SURGICAL TECHNIQUE

The operation is performed in two stages. The first stage involves the exteriorization of the ureters and their attachment to the skin just dorsal to the vent. The second stage involves complete isolation of the ureters from the cloaca. Approximately one week should be allowed between the first and second stages of the operation in order that a blood supply to the exteriorized portion can become established and thereby prevent sloughing of the tissue when the second stage is performed.

The initial, vertical, midline incision is made through the skin, perineal muscle, and the dorsal mucosa of the cloaca (fig. 1a). The incision is approximately 1 inch in length for the hen. The incised edges of the vent are retracted laterally and the incision is extended dorsally through the skin and perineal muscle (fig. 1b). The perineal muscle in this area is mostly aponeurotic tissue. The urodeum is then retracted posteriorly and the incision is extended anterolaterally on each side to a point just lateral to each ureteral opening (fig. 1c). Care must be taken in making this incision not to cut a ureter or to penetrate the peritoneum.

Simple interrupted mattress sutures, with No. 0 braided silk or No. 40 cotton thread, are used to unite the reflected mucosa of the urodeum (fig. 1d) to the skin just below the pygostyle. Sulfathiazole and penicillin crystals are sprinkled in the incision before closing. These sutures are removed after five days. The incised edges of the dorsal lip of the vent are not sutured. They are left as an open wound.

The second stage of the operation is performed two days after the sutures are removed. Starting near the midline, a tension suture is placed in the mucosa on each side, on a line midway between the ureteral openings and the opening of the oviduct (fig. 1e). Using these tension sutures for retraction, a transverse incision is made through the mucosa between the tension sutures and the opening of the oviduct (fig. 1e). The mucosa is reflected posteriorly approximately ⅛ inch by blunt dissection, again using care to avoid cutting a ureter or to penetrate the perito-

From the Departments of Veterinary Science and Poultry Industry, Louisiana Agricultural Experiment Station, Baton Rouge.

Published with the approval of the director of the Louisiana Agricultural Experiment Station.

The authors acknowledge the assistance of Hayden R. Williams in making the illustration.

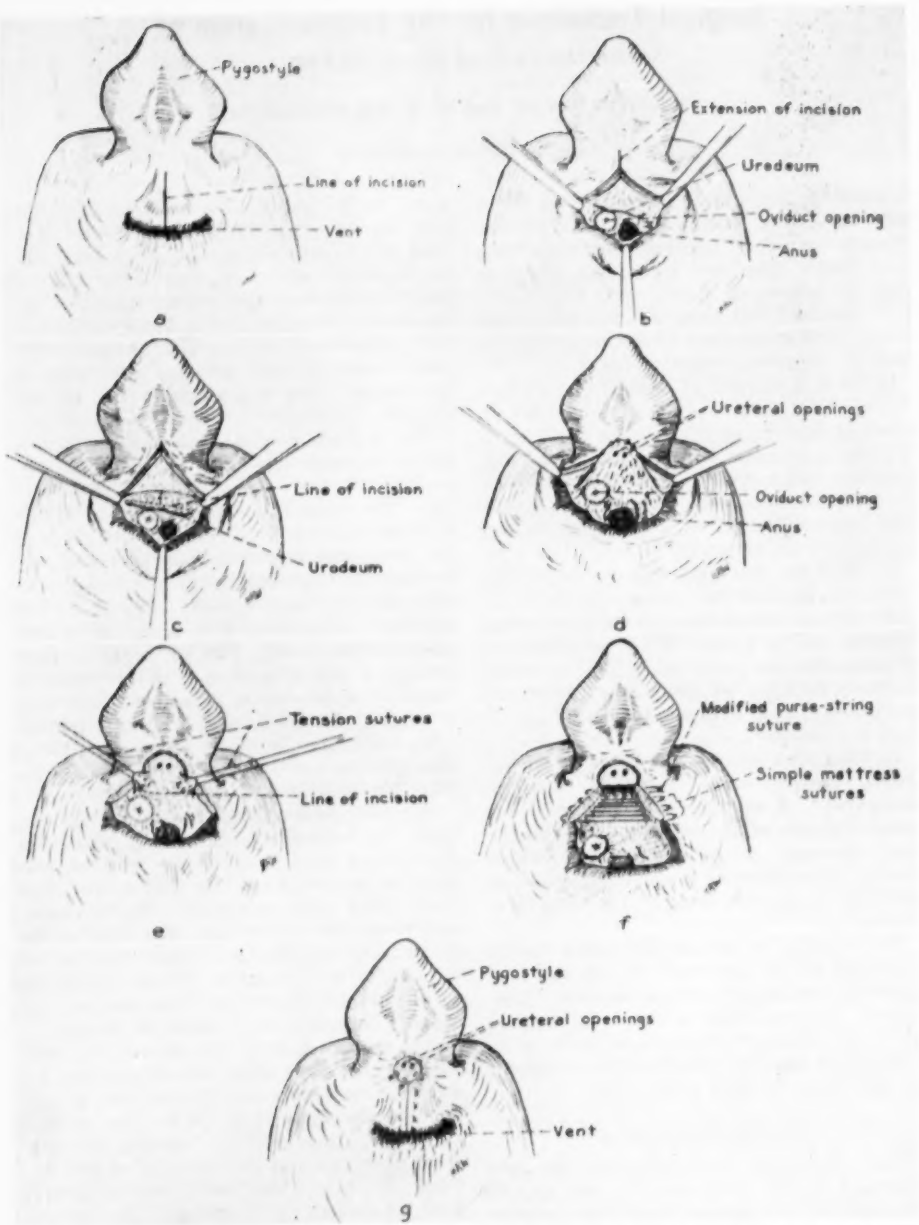


Fig. 1—Surgical technique for exteriorization of the ureters of the chicken: (a, b, c, and d) first stage and (e, f, and g) second stage.

neum. The mucosa containing the ureteral openings is now completely isolated from the mucosa of the cloaca.

To close the wound, the cut edges of the vent are freshened by the removal of all scar tissue. A modified purse-string suture is placed (fig. 1f) beginning on the right side, through the skin, perineal muscle, the mucosa of the cloaca (anal fold), perineal muscle, and skin. The suture is continued dorsally through the skin and perineal muscle across through the perineal muscle and skin of the right side where the knot is tied. Then, the wound in the dorsal lip of the vent is closed with simple interrupted mattress sutures (fig. 1f). As the sutures are tied the dorsal mucosa of the cloaca and the dorsal lip of the vent are reunited. Sulfathiazole and penicillin crystals are sprinkled in the incision before closing. Simple interrupted sutures are used to unite the isolated mucosa surrounding the ureteral openings to the skin (fig. 1g).

A urine collection apparatus, composed of a canvas harness, can now be put in place to hold the urine collection bag in place. This will hasten healing of the wound by protecting the incision from the urine. The urine collection bag is made from 2-inch (diameter) latex tubing. The feces are collected in dropping pans. The sutures are removed in five to seven days.

Summary

A method for the exteriorization of the ureters of the chicken is described and illustrated. This operation modifies the bird to permit quantitative collection of uncontaminated urine and feces for metabolism studies. This technique is applicable to chickens and other poultry. The operation does not interfere with normal laying.

References

[1] Minkowski, O.: Ueber den Einfluss der Leberexstirpation auf den Stoffwechsel. Arch. f. exper. Path. u. Pharmakol., *21*, (1886): 41 (cited by Hester *et al.*).

[2] Milroy, T. H.: The Formation of Uric Acid in Birds. J. Physiol., *30*, (1903): 47.

[3] Hester, H. R., Essex, H. E., and Mann, F. C.: Secretion of Urine in the Chicken *(Gallus Domesticus)*. Am. J. Physiol., *128*, (1940): 592–602.

[4] Sime, J. T., and Johnson, B. C.: Metabolism of Methionine $C^{14}H_3$ in the Chick. II. Distribution of C^{14} in the Urinary Uric Acid Molecule. J. Biol. Chem., *215*, (1955): 41–44.

[5] Hart, W. M., and Essex, H. E.: Water Metabolism in the Chicken *(Gallus Domesticus)* with Special Reference to the Role of the Cloaca. Am. J. Physiol., *136*, (1942): 657–668.

"Atomized" Distemper Vaccine of Avian Origin
II. Field Experience in Mink

D. W. JOHNSON, D.V.M.; V. J. CABASSO, Sc.D.;
K. HUFFMAN, B.S.; M. R. STEBBINS, M.S.

Pearl River, New York

THE PREVIOUS COMMUNICATION[1] reported experimental results obtained with either dust or aqueous "atomized" distemper vaccines of avian origin in ferrets and mink. Whereas the dust vaccine elicited no apparent response in ferrets, the aqueous spray afforded 100 per cent protection of the animals on challenge and was selected for a limited laboratory trial in mink. Although the over-all response, as measured by the complement-fixation (CF) method, was 59 per cent in animals of the latter species, the results were encouraging enough to warrant a further investigation of the method on a larger scale.

This communication reports the results of an aqueous atomized vaccine in mink under field trial conditions.

MATERIALS AND METHODS

Virus Strain.—The chicken embryo-adapted virus used in the preparation of experimental vaccines was the same as that reported in the accompanying paper.[1]

Experimental Vaccines.—Two lots of vaccine (6368-30A, trial 1, and 6368-34A, trial 2) were prepared as follows: an aqueous 40 per cent suspension by weight was made with infected embryonic tissues (chorioallantoic membranes and whole embryos). After centrifugation at 2,000 r.p.m. for 20 minutes, the clear supernatant was distributed in 33-ml. amounts in glass vials and dried from the frozen state. The vials were rubber-stoppered under vacuum, and stored at 4 C. until used. Bacterial sterility, animal safety, and potency tests were carried out as for commercial lots of vaccine.

Method of Application of Vaccine.—After reconstitution with sterile, distilled water, the vaccine was applied while the individual animal was trapped in a nest box. It was too late in the season to spray entire families before separation of the kits. "Atomizing" was by means of a DeVilbiss 40 nebulizer* with the side cork removed. The nebulizer was connected to a cylinder of water-pumped nitrogen, and the vaccine was expelled under a pressure sufficient to deliver 5 ml. in four to five minutes.

Equipment Required.—For optimal application of the atomized vaccine under ranch conditions, the equipment shown (fig. 1) was decided upon after consideration of factors important to the average mink rancher, such as mobility, cost, etc. It was found expedient to have on hand sufficient slides to trap about 60 mink at a time.

Evaluation of Results.—Since the method of challenge with virulent virus has not always been found reliable in determining the immunity of mink to distemper, results in these trials were evaluated by the CF method as described previously.[1] Blood specimens were secured by heart puncture before and after vaccination from a representative sample of the animals. The serum was separated from the clot on the day of bleeding and stored at 4 C. until tested.

EXPERIMENTAL PROCEDURES AND RESULTS

Ranch Trial 1 (Aug. 23–26, 1955).—This ranch had a total population of about 10,000 adult and young mink. Of these, 1,030 kits were vaccinated. They were of pastel and dark breeding, about 3 months of age, and had not been vaccinated previously.

Some time before the vaccination trial was initiated, the ranch started losing a few animals daily. Distemper infection was suspected to be present, as distemper virus was isolated from a single mink carcass early in the summer. However, a condition other than distemper, which remained unidentified despite serious laboratory efforts, was indicated by continuous moderate losses throughout the summer and fall.

Using a constant vaccine delivery rate, atomizing was carried out at four different time exposures: 60, 40, 20, and ten seconds per nest box. Two hundred and fifty animals were exposed for each of these intervals, and 30 additional kits received 1 ml.

From the Clinical Research and Viral and Rickettsial Sections, Research Division, American Cyanamid Co., Pearl River, N.Y.

The authors express their appreciation to Mrs. M. Murrin and Mr. H. Klaver for able technical assistance, and to Mrs. E. Osborn for help in the preparation of the manuscript.

[1] Cabasso, V. J., Johnson, D. W., Stebbins, M. R., and Cox, H. R.: "Atomized" Distemper Vaccine of Avian Origin. I. Experimental Immunization of Ferrets and Mink. Am. J. Vet. Res., *18*, (1957).

* The DeVilbiss Co.

of the same vaccine subcutaneously. The rest of the mink on the ranch, almost 9,000, were vaccinated subcutaneously by the owner with a commercially available preparation.

The nest boxes were of relatively tight construction and had no inside partition. The nose of the nebulizer was introduced into a hole drilled for the purpose on the face of the box, and it was possible to hold the nebulizer in a vertical position. At the time of application of the vaccine, the bottom and side openings of the boxes were closed by sheet-metal pieces cut to fit over these openings. After delivery of the vaccine, the atomizing holes were stoppered with corks, and the boxes were left tightly closed for at least ten more minutes. The interval between atomizing and postvaccination bleeding was six weeks. Of the approximately 25 animals from each time-exposure group which were bled before vaccination, 17 to 21 were bled after vaccination. Losses were caused by intercurrent infection on the ranch or by the bleeding procedure. In addition, seven postvaccination samples were obtained from animals vaccinated subcutaneously.

The results of this trial were favorable (tables 1 and 2). Blood specimens from the 60-, 40-, or 20-second exposure groups were 100 per cent positive, with substantial CF antibody titers, as were those of the subcutaneous group; whereas only 41 per cent from animals exposed for ten seconds became positive. The fact that 59 per cent of the animals of this latter group remained negative six weeks after application of the vaccine strongly suggests that the supposed distemper infection on the premises had little or nothing to do with the response of the mink kits in this trial.

Ranch Trial 2 (Nov. 1–2, 1955).—The purpose of this second trial was to confirm the findings reported above with regard to time of exposure and to determine whether the use of corks following exposure was necessary.

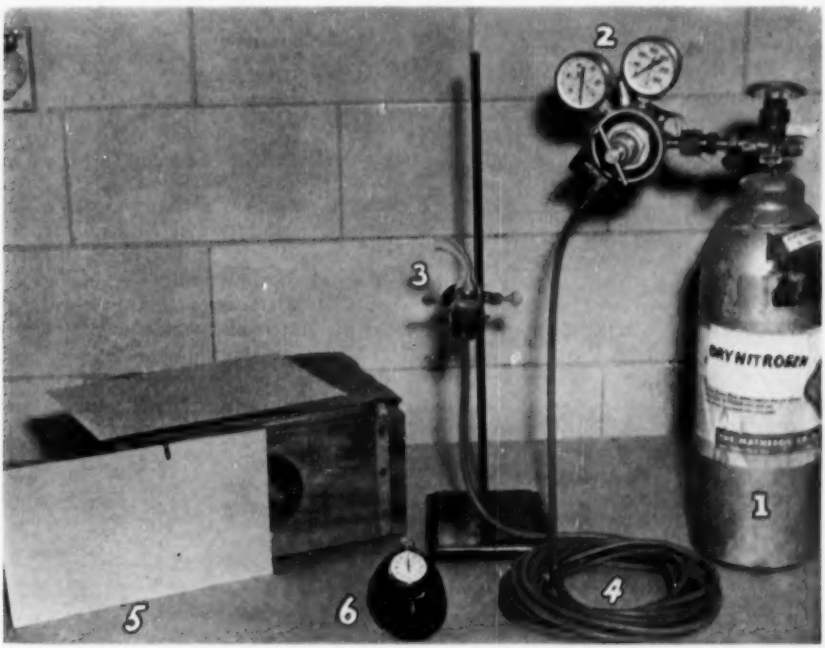


Fig. 1—Equipment used for application of "atomized" distemper vaccine.
(1) A cylinder of water- or oil-pumped nitrogen. (A 64-cu. ft. cylinder is sufficient for about 340 mink.) (2) Nitrogen cylinder regulator. (3) DeVilbiss 40 nebulizer containing vaccine. (4) Rubber tubing—vacuum type (50 ft.). (5) Sheet metal slides for closing nest box apertures. (6) Stop watch (watch with a sweep second hand can also be used).

TABLE 1—Ranch Trial 1—Complement-Fixation (CF) Response of Mink Kits Exposed to "Atomized" Aqueous Distemper Vaccine for Various Time Intervals

Exposure time (sec.)	No. exposed	Pre-exposure CF results				No. dead of intercurrent infections or bleeding	No. bled 6 weeks postexposure	Postexposure CF results		
		No. tested	Pos.	Neg.	AC*			Pos.	Neg.	Pos.(%)
60	250	25	—	24	1	2	21	21	—	100
40	250	23	—	23	—	4	19	19	—	100
20	250	24	—	21	3	—	17	17	—	100
10	250	24	1	23	—	4	17	7	10	41
Subcutaneous vaccination	30	20	—	19	1	4	7	7	—	100

* Serum anticomplementary.

The mink kit population of this ranch consisted of 510 animals of both sexes, about 5 months of age. Eighty per cent were sapphires, 10 Aleutians, 5 dark, and 5 pastels. To the owner's knowledge, there had been no distemper infection on the ranch in recent years and no vaccine had been used there since the kits were born.

Atomizing was done as in the previous trial, except that only three time exposures were used and, following delivery, the atomizing holes of only half the nest boxes were plugged with corks. Details pertaining to the setting up of this trial are given (table 3).

The random prevaccination sampling was intended to ascertain the absence of CF antibodies in this age group. Animals of group 8 were left unvaccinated for purposes of comparison, so that the owner could see whether vaccination at this time of year would have an adverse effect on the furring-out and priming of the animals before pelting. The subcutaneously vaccinated group was included for proof of potency of the vaccine.

Because of a bleeding factor in the sapphire strain of mink, an arrangement was made with the owner that postvaccination blood samples would be secured only at the time of pelting, so as not to lose an undue number of animals by heart puncture. As it was, 11 kits died as a result of prevaccination bleeding. Because of the early onset of cold weather, pelting took place sooner than usual and most of the postvaccination testing was done at 30 days. As shown in table 3, a total of 155 blood specimens was obtained at the time of pelting, all from the sapphire strain—144 were taken at 30 days and 11 at 45 days. Pre- and postvaccination samples were tested for distemper antibodies by the CF method.

All prevaccination bleedings were negative. Results on postvaccination samples (table 4) indicate that the vaccine was of satisfactory potency, since the 3 animals inoculated subcutaneously were positive.

TABLE 2—Ranch Trial 1—Complement-Fixation (CF) Titer Range of Mink Kits Six Weeks After Exposure to "Atomized" Aqueous Distemper Vaccine

Exposure time (sec.)	No. tested	CF titers					
		< 1:4	1:8	1:16	1:32	1:64	1:128
60	21	—	1	3	9	5	3
40	19	—	1	—	2	4	12
20	17	—	—	1	4	8	4
10	17	10	—	2	—	2	3
Subcutaneous vaccination	7	—	—	—	2	4	1

TABLE 3—Distemper Vaccination and Bleeding Schedule of Mink Kits in Ranch Trial 2

Group No.	No. of kits	Mode of vaccination	No. of kits bled prevaccination	Exposure time (sec.)	Corks used	No. of serum samples tested postvaccination
1	50	Spray	10	10	Yes	23
2	50	Spray	10	10	No	17
3	50	Spray	10	20	Yes	22
4	50	Spray	10	20	No	39
5	140	Spray	10	40	Yes	36
6	50	Spray	10	40	No	15
7	10	1 ml. subcutaneously	3	—	—	3
8	110	None	None	—	—	—

TABLE 4—Ranch Trial 2—Complement-Fixation (CF) Response of Mink Kits Exposed to "Atomized" Aqueous Distemper Vaccine

Exposure time (sec.)	Corks used	No. tested	CF results		
			Pos.	Neg.	Pos.(%)
10	Yes	23	11	12	48
10	No	17	1	16	6
20	Yes	22	15	7	68
20	No	39	31	8	80
40	Yes	36	23	13	64
40	No	15	7	8	47
Totals		152	88	64	59
Disregarding 10-sec. group		112	76	36	68
Subcutaneous vaccination		3	3	—	100

The response to atomized vaccine, however, varied between 80 per cent (20 seconds, no corks) and 6 per cent (10 seconds, no corks), suggesting that the use of corks is not necessary, providing an adequate exposure time is employed. When calculated on the basis of total kits sprayed, the overall CF response was 59 per cent. This figure includes animals given the 10-second exposure time, which was shown to be insufficient in ranch trial 1. By omitting this latter group of animals from calculation, the percentage of positive specimens increases to 68.

It was of interest in this trial that vaccination in November, whether by atomized vaccine or by the subcutaneous route, had no adverse effect on the quality of the fur when vaccinated and unvaccinated mink were compared.

Discussion

The vaccination of mink under field conditions with an atomized aqueous vaccine was found practical for rapidity and convenience of administration and, using the CF response as the criterion for acquired resistance, to a large extent effective. Although the method had given 100 per cent protection to ferrets under experimental conditions, its immunizing potential seems less predictable on the mink ranch. Whereas all the animals exposed for over 20 seconds exhibited positive CF titers in the first trial, only 68 per cent did so in the second. However, immunity to distemper may not necessarily be associated with the presence of CF antibodies. But that they do constitute a legitimate measurement of immunity seems to derive from the fact that all animals vaccinated subcutaneously gave positive CF results.

The discrepancies observed between the findings in the two ranch trials may in part have been caused by the fact that the experimental herds involved belonged to different strains and that the time of post-exposure bleeding may not have been optimum in the second trial. Also, the nature of this vaccination procedure is such that there may always be unforeseen variations in field use. Despite its limitations, however, the atomizing method may be of value in establishing a substantial herd immunity, providing it is used under specified conditions.

The time of exposure to the vaccine mist is of great importance: 20 seconds, under the conditions of these trials, appears to be the minimum. This suggests the necessity of spraying in multiples of 20-second periods should a mother and her litter be simultaneously atomized in the same nest box. The physical condition of the box is also of importance. It should be of reasonably tight construction, free of large cracks and holes. The necessary apertures should be conveniently reached for fitting with sheet-metal slides at the time of spraying. The size of the atomizing hole should be kept at a minimum, just large enough to accommodate the nose of the nebulizer. The latter should be kept in a vertical position during application of the vaccine for optimal delivery and to avoid leakage. Finally, atomizing must be carried out at temperatures above freezing to avoid ice formation in the capillary tubes within the nebulizer.

Summary

An aqueous "atomized" distemper vaccine of avian origin was evaluated in a total of 1,390 mink on two separate ranches and was found capable of eliciting complement-fixing antibodies in 68 to 100 per cent of animals following a single application.

A minimum exposure time of 20 seconds per animal was found necessary when the vaccine was applied under nitrogen pressure by means of a DeVilbiss 40 nebulizer at a delivery rate of 5 ml. of vaccine in four to five minutes.

Ringworm in Wild Animals in Southwestern Georgia

ROBERT W. MENGES, D.V.M., M.P.H.; GORY J. LOVE, B.S.;
WILLIAM W. SMITH, Ph.D.; LUCILLE K. GEORG, Ph.D.

Atlanta and Newton, Georgia

In August, 1954, a study was initiated to determine the prevalence and the etiological agents of ringworm in wild animals in southwestern Georgia. The purpose of this paper is to present the findings accumulated during the period of Aug. 26, 1954, to Sept. 23, 1955.

Most of the reports concerning ringworm in wild animals have been descriptions of isolated cases or epizootics. Menges and Georg [1] have summarized the literature on wild animal isolations of dermatophytes; exclusive of captive animals, only two organisms have been recovered: *Trichophyton mentagrophytes* from foxes, mice, muskrats, rabbits, and squirrels, and *Trichophyton schoenleini* from mice.

This report presents a survey of apparently normal wild animals. Only 16 of the 1,142 animals examined had visible skin lesions. The lesions were all observed on Norway rats and no dermatophytes were obtained from these animals.

Materials and Methods

Hair specimens were obtained from animals trapped by the Communicable Disease Center's Newton Field Station at Newton, Ga. With sterile forceps, hairs were plucked arbitrarily from the nose and back of the animals and placed in clean envelopes. These were mailed to the Mycology Unit in Chamblee, Ga., where the specimens were checked with a Wood's lamp for fluorescence and, after treatment with 10 per cent potassium hydroxide, a direct microscopic examination for fungous elements was made. Following these examinations, other hairs from each specimen were placed on cycloheximide medium [2] and the cultures were held at 25 C. for a minimum of one month before being discarded as negative.

Soil specimens were obtained from some of the areas where animals were trapped. The specimens were cultured by the hair-baiting technique developed by Vanbreuseghem [3] and used in the successful isolation of *Microsporum gypseum* from soil by Ajello.[4] The samples were gathered from the upper surface of the collection site by scraping the soil directly into 4-oz. bottles.

From the Communicable Disease Center, Bureau of State Services, Public Health Service, U. S. Department of Health, Education, and Welfare, Atlanta and Newton, Ga.

Results

In the survey, hair specimens from 1,142 wild animals of 21 species were cultured and dermatophytes were isolated from 88 (7.7%, table 1). None of the hair specimens showed fluorescence, and none were positive on direct microscopic examination.

Fig. 1—*Microsporum gypseum* macroconidia. x 475.

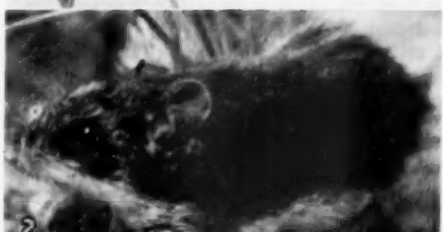


Fig. 2—Old-field mouse (Peromyscus polionotus).

TABLE 1—Wild Animals from Which Hair Specimens Were Cultured and Number and Percentage Positive with the Organisms Isolated

Animal	No. examined	Positive		Organism isolated		
		No.	%	*Microsporum gypseum*	*Microsporum gypseum* (red variety)	*Trichophyton mentagrophytes*
Opossum (*Didelphis virginiana*)	57	1	1.7	....	....	1
Red bat (*Lasiurus borealis*)	1	0	0	....	....	....
Raccoon (*Procyon lotor*)	42	0	0	....	....	....
Striped skunk (*Mephitis mephitis*)	2	0	0	....	....	....
Red fox (*Vulpes fulva*)	2	0	0	....	....	....
Gray fox (*Urocyon cinereoargenteus*)	5	0	0	....	....	....
House cat (*Felis domestica*)	4	0	0	....	....	....
Bob cat (*Lynx rufus*)	2	0	0	....	....	....
Eastern gray squirrel (*Sciurus carolinensis*)	1	0	0	....	....	....
Eastern fox squirrel (*Sciurus niger*)	6	0	0	....	....	....
Harvest mouse (*Reithrodontomys humulis*)	21	3	14.3	....	3	....
Old-field mouse (*Peromyscus polionotus*)	273	27	9.9	2	13	12
Cotton mouse (*Peromyscus gossypinus*)	53	19	35.8	....	17	2
Golden mouse (*Peromyscus nuttalli*)	5	1	20.0	....	1	....
Pine mouse (*Pitymys pinetorum*)	1	0	0	....	....	....
House mouse (*Mus musculus*)	191	3	1.6	....	2	1
Cotton rat (*Sigmodon hispidus*)	23	8	34.8	....	7	1
Woodrat (*Neotoma floridana*)	14	4	28.6	....	4	....
Norway rat (*Rattus norvegicus*)	350	17	4.8	2	....	15
Roof rat (*Rattus rattus*)	84	5	6.0	....	....	5
Eastern cottontail rabbit (*Sylvilagus floridanus*)	3	0	0	....	....	....
Total	1,142	88	7.7	4	47	37

TABLE 2—Number and Percentage of Rats Infected with Ringworm According to Age

Animal	Age														
	Young			Subadult			Adult			Unknown			Total		
	No.	+	%	No.	+	%	No.	+	%	No.	+	%	No.	+	%
Norway rat (*Rattus norvegicus*)	11	2	18.2	58	3	5.2	242	10	4.1	39	2	5.1	350	17	4.9
Roof rat (*Rattus rattus*)	5	0	0	8	2	25.0	71	3	4.2	0	0	0	84	5	6.0
Total	16	2	12.5	66	5	7.6	313	13	4.2	39	2	5.1	434	22	5.1

No. = number of rats examined; + = number positive; % = percentage positive.

TABLE 3—Wild Animals from Which Hair Specimens Were Cultured and Number and Percentage Positive According to Sex

Animal	Sex					
	Male			Female		
	No. examined	Positive		No. examined	Positive	
		No.	%		No.	%
Opossum (*Didelphis virginiana*)	31	0	0	28	1	3.6
Red bat (*Lasiurus borealis*)	....	....	....	1	0	0
Raccoon (*Procyon lotor*)	19	0	0	23	0	0
Striped skunk (*Mephitis mephitis*)	....	....	....	2	0	0
Red fox (*Vulpes fulva*)	....	....	....	2	0	0
Gray fox (*Urocyon cinereoargenteus*)	3	0	0	2	0	0
House cat (*Felis domestica*)	....	....	....	4	0	0
Bob cat (*Lynx rufus*)	2	0	0	....	....	....
Eastern gray squirrel (*Sciurus carolinensis*)	1	0	0	....	....	....
Eastern fox squirrel* (*Sciurus niger*)	3	0	0	1	0	0
Harvest mouse (*Reithrodontomys humulis*)	7	0	0	14	3	21.4
Old-field mouse (*Peromyscus polionotus*)	109	16	14.7	164	11	6.7
Cotton mouse (*Peromyscus gossypinus*)	30	9	30.0	23	10	43.5
Golden mouse (*Peromyscus nuttalli*)	....	....	....	5	1	20.0
Pine mouse (*Pitymys pinetorum*)	....	....	....	1	0	0
House mouse (*Mus musculus*)	73	0	0	118	3	2.5
Cotton rat (*Sigmodon hispidus*)	13	2	15.4	10	6	60.0
Woodrat (*Neotoma floridana*)	8	2	25.0	6	2	33.3
Norway rat (*Rattus norvegicus*)	197	10	5.1	153	7	4.6
Roof rat (*Rattus rattus*)	34	1	2.9	50	4	8.0
Eastern cottontail rabbit (*Sylvilagus floridanus*)	2	0	0	1	0	0
Total	532	40	7.5	608	48	7.9

* Sex unknown of 2 animals.

Three different organisms were isolated: *M. gypseum* (fig. 1) from old-field mice (fig. 2) and Norway rats; *M. gypseum*, "red variety" (fig. 3), from harvest mice, old-field mice, cotton mice, golden mice (fig. 4), house mice, cotton rats (fig. 5), and woodrats; and *T. mentagrophytes* (fig. 6) from opossum, old-field mice, cotton mice, house mice, cotton rats, Norway rats, and roof rats.

The red variety of *M. gypseum* differed from the typical *M. gypseum* in that the macroconidia had thicker walls, and the colony developed a deep blood-red pigment in the agar. A similar organism was isolated from soil obtained from Idaho and Washington by Cooke,[5] from Michigan by Ajello,[9] and from Georgia by Menges and Georg.[6]

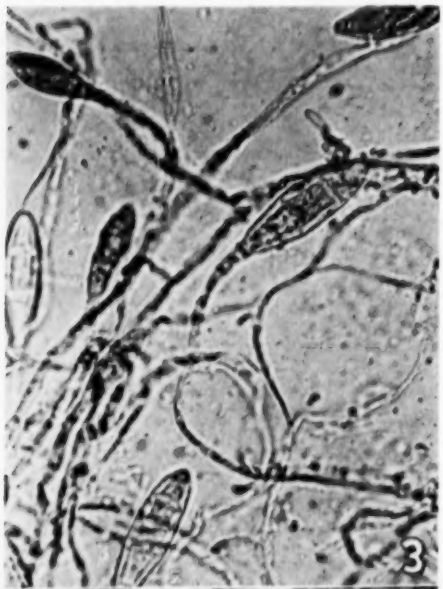


Fig. 3—Microsporum gypseum (red variety) macroconidia. x 475.

Fig. 4—Golden mouse (Peromyscus nuttalli).

Data concerning the age of the animals were obtained only for 350 Norway rats and 84 roof rats (table 2). The percentage positive decreased with age from 12.5 in young rats to only 4.2 in adult rats.

Of the 1,142 animals examined, 532 were males and 608 were females; the sex of 2 was unknown (table 3). Among the species in which the numbers examined were adequate for a comparison, the findings were variable. There appeared to be no difference in the infection rate of the sexes of the Norway rats, but a marked difference of infection (14.7% males, 6.7% females) among the old-field mice.

The seasonal distribution of ringworm in these wild animals is shown (table 4). The seasonal peak (13.5%) occurred during the spring months (March, April, and May). A comparison of the seasonal peaks of three groups, (1) old-field mice, (2) Norway rats, and (3) all species, indicated that the peak month for all of the species was in April (20.8%), with another lower peak (13.0%) in July. The old-field mice had a peak in April and another in August. The Norway rats showed low peaks in February, April, May, and July. Population studies done prior to the ringworm survey indicated that feral rodent populations reached peak proportions about one year prior to the beginning of the survey.

The location of the wild animal ringworm cases in Georgia is shown (table 5). When all species were included, the percentage positive was similar for the four counties where most of the animals were trapped; however, there was some variation in the distribution of infection in the rats and mice.

The habitats in which 672 of the wild animals were trapped are shown (table 6). A comparison of the habitats is not possible because adequate numbers were not sampled in all categories.

When the number of years since fields were cultivated was compared, it was

TABLE 4—Wild Animals from Which Hair Specimens Were Cultured and Number and Percentage Positive, with the Organisms Isolated According to Season

Season *	No. examined	Positive		Organism isolated		
		No.	%	*Microsporum gypseum*	*Microsporum gypseum* (red variety)	*Trichophyton mentagrophytes*
Winter	265	16	6.0	0	5	11
Spring	258	35	13.5	2	24	9
Summer	249	20	8.0	2	10	8
Fall	370	17	4.5	0	8	9
Total	1,142	88	7.7	4	47	37

* Months included: winter, December to February; spring, March to May; summer, June to August; and fall, September to November.

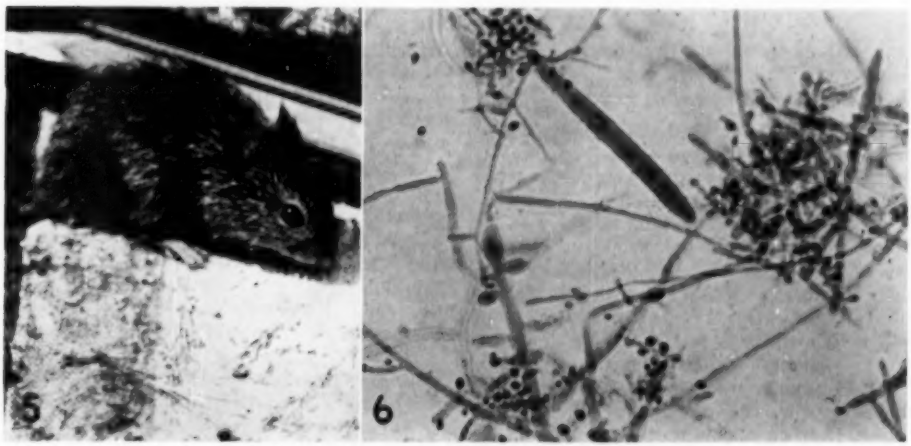


Fig. 5—Cotton rat (Sigmondon hispidus).
Fig. 6—Trichophyton mentagrophytes microconidia and macroconidia. x 475.

found that the percentage of infection in animals trapped in the older fields was somewhat greater, but not markedly different.

The habitats from which *M. gypseum* was isolated from soil in three counties in Georgia are shown (table 7). A total of 62 soil specimens were cultured, with 16 positive (26.0%). Most of the isolations were from soil collected under and around barns. All of the isolates were the typical *M. gypseum*. *Keratinomyces ajelloi* was isolated from one soil specimen from under a house.

It is of interest that on one farm in Mitchell County where the red variety of *M. gypseum* was isolated from 18 (27.6%) of 65 rodents trapped, the soil specimens

TABLE 5—Location of Surveys for Ringworm Infection of Wild Animals in Georgia

County	Animal								
	All species			Rats			Mice		
	No.	+	%	No.	+	%	No.	+	%
Baker	640	47	7.3	58	9	15.5	478	37	7.7
Mitchell	217	18	8.3	146	2	1.4	57	16	28.1
Grady	212	18	8.5	202	18	8.9	8	0	0
Thomas	61	5	8.2	61	5	8.2	0	0	0
Decatur, Miller, Dougherty	12	0	0	4	0	0	1	0	0
Total	1,142	88	7.7	471	34	7.2	544	53	9.7

No. = number examined; + = number positive; % = percentage positive.

TABLE 6—Habitats Where 672 Wild Animals Were Trapped

Type of habitat	No. examined	Positive		Organism isolated		
		No.	%	*Microsporum gypseum*	*Microsporum gypseum* (red variety)	*Trichophyton mentagrophytes*
Fields *	370	24	6.4	0	12	12
Bottomland	48	6	12.5	0	5	1
Bird feed patch	15	4	26.7	2	0	2
Woods (pine, oak)	95	17	17.9	0	17	0
Barn (mule, dairy)	64	7	10.9	2	2	3
Swamp and milo field	61	8	13.1	0	6	2
Old road	19	1	5.3	0	0	1
Total	672	67	9.9	4	42	21

* Includes corn, lupine, dog fennel, peanut, weed, broomsedge, meadow grass, brush and briar, astor weed, and open fields.

from 20 areas were cultured and found negative.

DISCUSSION

This survey of wild animals revealed that ringworm infection caused by *T. mentagrophytes* is apparently common among rodents. It is of interest that these animals harbor this fungus without demonstrating any clinical signs of infection and may be a source of *T. mentagrophytes* infection in man in rural areas. Human beings do not need to have direct contact with rats in order to acquire infection, since dermatophytes on hairs or skin scales may remain viable for many months and certain areas of farm premises, especially feed bins and barns, may be contaminated by spores and infected hairs shed by rodents. Because of their ubiquity and abundance, rodents would constitute a more likely source of human infection than the occasional infected dog or other farm animal.

Trichophyton mentagrophytes has been isolated from soil on only three occasions[7,8] and, in these instances, hairs from infected animals may have been present.

TABLE 7—Habitats from Which Microsporum Gypseum Was Isolated from Soil in Baker, Mitchell, and Grady Counties, Georgia

Type habitat	No. of soil specimens cultured	Positive (*Microsporum gypseum*)	
		No.	%
Uncultivated field	16	0	0
Planted pine trees	4	0	0
Pine and broomsedge field	6	0	0
Thick oak-pine woods	6	0	0
Around barns	10	6	60
Under barns	8	6	75
Smoke house	4	1	25
Vacant garage	2	0	0
Under houses *	6	3	50
Total *	62	16	26

* *Keratinomyces ajelloi* was also isolated from one of the soil specimens.

In cultural studies of 439 soil samples,[9] *T. mentagrophytes* has never been isolated. Thus, there is little evidence to suggest that this fungus exists as a saprophyte in nature.

Microsporum gypseum is a common soil saprophyte[9] and infections would be expected in wild animals. The red variety of *M. gypseum* was isolated most frequently from the wild animals, but only the typical *M. gypseum* was isolated from the soil. However, the red variety has been isolated from soil in other areas.[6]

Further studies of wild and domestic animals throughout the United States would aid materially in the understanding of the epidemiology of ringworm. These studies may eventually point the way toward the control of ringworm in man and animals.

SUMMARY

In a survey of 1,142 wild animals of 21 species for ringworm, dermatophytes were isolated from hair specimens of 88 (7.7%) of the animals. The organisms commonly isolated were *Microsporum gypseum* from *Peromyscus polionotus* and *Rattus norvegicus; M. gypseum* ("red variety") from *Reithrodontomys humulis, P. polionotus, Peromyscus gossypinus, Peromyscus nuttalli, Mus musculus, Sigmodon hispidus,* and *Neotoma floridana;* and *Trichophyton mentagrophytes* from *Didelphis virginiana, P. polionotus, P. gossypinus, M. musculus, S. hispidus, R. norvegicus,* and *Rattus ratus.* Infection was more common in young rats (12.5%) than in old rats (4.2%), and there was little difference in the infection rate in sexes. The seasonal peak (13.5%) occurred during the spring months. The peak month for all of the species was in April (20.8%) with another lower peak

(13.0%) in July. The distribution of infection was similar for the four counties in southwestern Georgia where most of the animals were trapped, with some variation in the rats and mice. Sixty-two soil specimens from areas where animals were trapped were cultured and *M. gypseum* was isolated from 16 (26.0%).

References

[1] Menges, R. W., and Georg, L. K.: Animal Ringworm Survey. Vet. Med., *50*, (1955): 293–297.

[2] Georg, L. K., Ajello, L., and Papageorge, C.: Use of Cycloheximide in the Selective Isolation of Fungi Pathogenic to Man. J. Lab. and Clin. Med., *44*, (1954): 422–428.

[3] Vanbreuseghem, R.: Technique biologique pour l'isolement des dermatophytes du sol. Ann. Soc. Belge de méd. trop., *32*, (1952): 173–178.

[4] Ajello, L.: The Dermatophyte, *Microsporum Gypseum*, as a Saprophyte and Parasite. J. Invest. Dermat., *21*, (1953): 157–171.

[5] Cooke, W. B.: Western Fungi. II. Species from Eastern Washington and Adjacent Idaho. Mycologia, *44*, (1952): 245–261.

[6] Menges, R. W., and Georg, L. K.: Canine Ringworm Caused by *Microsporum Gypseum*. Cornell Vet., *47*, (1957): 90–100.

[7] Muende, I., and Webb, P.: Ringworm Fungus Growing as a Saprophyte Under Natural Conditions. Arch. Dermat. and Syph., *36*, (1937): 987–990.

[8] Lurie, H. I., and Borok, R.: *Trichophyton Mentagrophytes* Isolated from the Soil of Calves. Mycologia, *47*, (1955): 506–510.

[9] Ajello, L.: Soil as Natural Reservoir for Human Pathogenic Fungi. Science, *123*, (1956): 876–879.

The Production and Use of Germ-Free Animals in Experimental Biology and Medicine

JAMES A. REYNIERS

Notre Dame, Indiana

THE PURE CULTURE CONCEPT has been most closely identified with microbiology. However, in its essentials, this concept is basic to all experimental science whether it be physical or biological. The need for isolating "pure units" from the natural complex in which they exist forms the basis of analysis and the combination of pure units to build back to a predetermined level is identified with synthesis. Whether these pure units are compounds, physical particles, bacteria, animals, or mathematical symbols does not alter the philosophy.

All animals are, from birth, a complex or combination of parasites (contaminants) and the host (animal). As such, the conventional animal is an "impure unit."

The interaction of the host and contaminant systems throughout the life of the conventional animal must be accounted for, in the last analysis, with respect to biological functions which measure the success of a species in time.[1] These things are difficult to assay with certainty because little is known about the animal in the absence of all contaminants and little control can be exerted on the contamination as it naturally occurs.

A living being is an intricate and dynamic complex of ancestral promptings, maternal influences, and individual experiences gained from the environment into which it is born and must live.[2] The experience gained from contact with living contaminants by the animal throughout its life is profound; however, the exact impact of these contaminants is obvious only where the results are measurable, as in disease and death. The more subtle implications involving the activation of special defensive systems with which the animal is endowed, the reflection of this activation upon other host systems not directly concerned with defense, and the interaction between the microbial components themselves are not so obvious nor can they be easily measured unless the "pure animal" is considered.

In seeking to reduce this dynamic complex to a degree of purity consistent with the level of inquiry and the phenomenon, a first step can be taken by separating the host from its contaminants and observing it through life as a "germ-free" animal. Should a greater degree of purity be indicated, it is then possible to rely upon genetics as a means of directing the germ-free animal through generations to a further state of uniformity and, in turn, to employ still other means of controlling the physical environment, nutrition, and association phenomena. By first obtaining and then studying germ-free life systematically, it is possible to arrive at a knowledge of what life is like in the same way that a pure culture of bacteria can be studied in a test tube. If then, as a next step, it is desirable to combine pure units, e.g., the germ-free animal and pure cultures of bacteria, this too may be accomplished in a controlled manner until the complex, as it exists in nature or at a wanted experimental level, is built up.

The specific uses to which germ-free life has been put will be considered later but, at this point, it might be well to consider the two main paths to be followed: (1) the study of germ-free life *per se* as a "pure culture" of rats, mice, or any other species or as a pure animal, and (2) the use of germ-free animals and techniques in experimental studies where the animal is the "medium."

1) The study of the germ-free animal

Dr. Reyniers is research professor of bacteriology and director, Lobund Institute, University of Notre Dame, Notre Dame, Ind.

These studies were aided by a contract between the Office of Naval Research, Department of the Navy, and the University of Notre Dame, NR131-067; by U. S. Public Health Service grant A-566; by the Damon Runyon Memorial Fund grant 48; and by contract with Surgeon General's Office, Department of the Army.

The author gratefully acknowledges the contributions of his associates, P. C. Trexler, R. F. Ervin, H. A. Gordon, M. Wagner, and B. S. J. Wostmann, and the assistance of B. A. Teah, J. R. Pleasants, and A. N. Lorenc of the Lobund Institute staff.

Presented before the Combined Sections on Public Health and Research, Ninety-Third Annual Meeting, American Veterinary Medical Association, San Antonio, Texas, Oct. 15–18, 1956.

per se is important in establishing base lines for experimental studies and in mapping out the more fruitful areas for further investigation. For example: since all animals are endowed at birth with defensive systems against living contaminants as well as other systems necessary to assure their life and continuity through successive generations, the development or lack of development of these systems may be studied in the absence of microbial impact. Nutrition may so be considered in a host which does not have to feed its contaminants.

2) The use of germ-free animals and techniques has been well established in experimental studies dealing with host-contaminant relationships in the etiology of dental caries,[3] pathogenesis of *Endamoeba histolytica*,[4] radiation sickness,[5] hemorrhagic shock, intestinal synthesis, the production of disease-free stocks of domestic and laboratory animals,[6] and with viruses as etiological and pathological agents. The spectrum of uses is as wide as biology.

It has become obvious that applied studies must rest on a knowledge of the germ-free animal *per se*, even in the face of controls run within the framework of the experiment and in the germ-free state. For example, it would seem a simple experiment to feed germ-free animals a diet which will cause caries in the conventional animal and observe the results. However, unless it is known how teeth develop in the germ-free state, there may be errors in interpreting the results. Also, the digestive tract of a germ-free animal is an organ of digestion and elimination across which the nutritive elements pass to the body of the animal. As such it is a thin-walled organ with little capillarization and connective tissue, and lacking in lymphoid elements. Although "stripped to its essential elements,"[7] this organ will adequately supply the needs of the germ-free animal. On the other hand, the digestive tract of the conventional animal serves the above functions, but also is an organ of containment for the microorganisms which grow in it.[8] The conventional gut tract is heavier, the walls thicker, and the lymphoid elements active. In setting up an experiment where the findings of the conventional state need further elaboration, these differences may or may not be important, but this importance will never be properly assayed until the function of the tract has been established for the germ-free state.

These are the broader aspects of the thesis upon which the Lobund germ-free life studies have rested since their inception in 1928.

PRODUCTION OF GERM-FREE ANIMALS

A germ-free animal is one which is known to be free from life other than that produced by its own protoplasm and which exists in a sterile environment.

1) *Obtaining Germ-Free Animals.*—Healthy animals, mammals or birds, may be free from living contaminants before birth or hatching. Biologically, this is a reasonable assumption which has been borne out through experiment since 1895,[9-20] both through attempts to obtain germ-free animals and in the use of embryos for virus cultivations.

As a fertilized ovum and through the period of gestation while the embryo is organizing the systems with which it will have to defend itself after birth, it is reasonable that it should be protected from infective microorganisms, and it is protected. This does not mean that infective agents may not pass the placental barrier or otherwise enter the egg before it is laid, but it also happens that the young from healthy mothers may be free from contamination up to birth.

Therefore, to obtain a germ-free animal, it is necessary to deliver mammals by cesarotomy and to hatch birds from eggs with sterilized shell surfaces. When contamination occurs, such animals are discarded. This first generation of animals can be permitted to grow to maturity, breed, care for their young, and continue to build a colony. Thus, they may be examined continuously at all ages and through generations for contamination. It is reasonable to expect that if microorganisms are present they will manifest themselves at some age or stage of the study.

Testing for contamination rests essentially on the basis that if contamination occurs within a germ-free unit all animals will be equally contaminated; and that contamination may be detected by microscopic examination, by cultural techniques, by pathological changes, serological tests, blind passages, tissue culture, and experience with the problem. The techniques employed are sufficient to assure that an animal called germ-free is free from ecto- and endoparasites, bacteria, yeasts, fungi, protozoa, viruses, and other forms of life available to testing. There may be some

TABLE 1—Five-Year Operation Lobund Institute—Species and Numbers of Animals Raised Postweaning *

Year	Rats	Mice	Chickens	Turkeys	Dogs	Rabbits	Guinea pigs
1950–1951	17	0	86	0	0	3	0
1951–1952	34	0	233	0	0	1	11
1952–1953	177	0	298	0	0	0	62
1953–1954	120	0	195	26	14	0	154
1954–1955	122	0	292	0	0	0	193
1955–1956	404	217	247	0	0	0	191
1956–1957	613	389	320	0	0	10	220

In the above table, the years showing "0" mean that no attempt was made to produce the species designated. The number of germ-free units available for this purpose is small (2 to 12); consequently, in certain periods, one species or another may be emphasized. The number of animals produced includes all ages through weaning; consequently, the numbers mentioned above will vary with the length of time they must be held.

* Data compiled by B. A. Teah, chief, Germ-Free Life Production, Lobund Institute.

question concerning such things as "inapparent viruses" or proviruses, but this is a special problem and involves "forcing" techniques which constitute a new area of research. For viruses which are detectable by pathological changes or serological tests, the answer is more certain.

Animals of the following species have been obtained germ free: monkey,[18] goat,[13] dog,[18] cat,[18] rat,[17,19] rabbit,[18] hamster, mice,[21] chickens,[11,12,16,20] turkeys,[21] guinea pigs.[10,13,14,22] This list probably could be extended greatly.

2) *Raising Germ-Free Higher Animals.*—It is one thing to obtain germ-free animals and another to raise them through maturity and into successive generations. Unless such animals can be held for long periods of time and in sufficient numbers to warrant their study and use systematically, their value is distinctly limited. Further, their value is extended if they can be bred into a colony which may then be standardized as to genetics and other desirable characteristics.

Many problems beset the investigator in this field. There are few investigators and much is unknown in such areas as the nutritional requirements of the suckling small laboratory mammal. Germ-free animals must be provided with sterilized food, water, bedding, cages, air, utensils through their entire lives. Suckling mammals must be provided with an artificial liquid diet which is hand-fed until they are weaned. Weaned animals are fed sterilized semi-

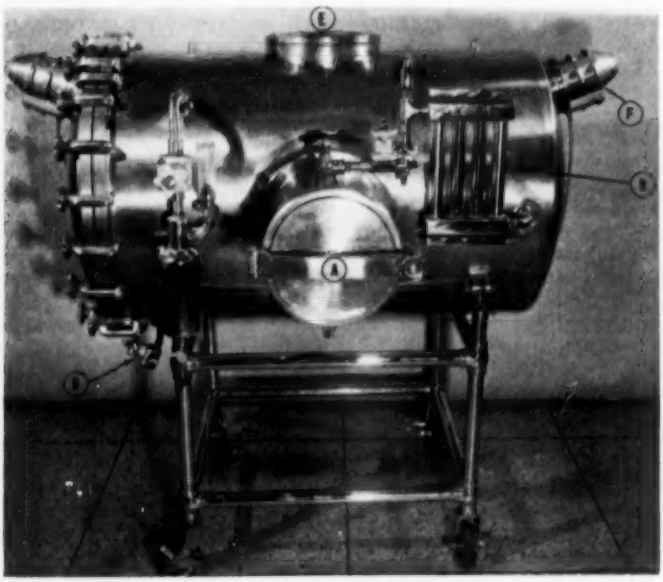


Fig. 1—Back view of the Reyniers standard germ-free unit. (A) Sterile lock or food clave, (B) air filters, (C) outlet trap for air, (D) main drain, (E) viewing port, and (F) external lamp housing.

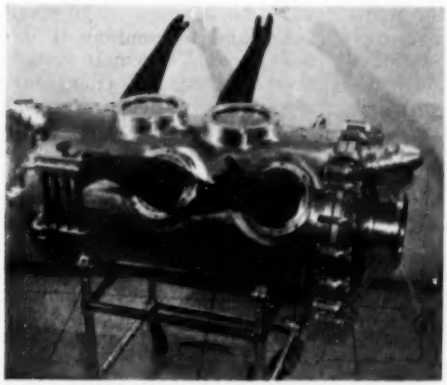


Fig. 2—Standard examining unit REXU-200. This is a two-man unit made to connect to an RSU-400 or other unit and is used principally for performing experiments on the animals.
(A) Connecting link to sterile lock or food clave. This is equipped with a standard quick-opening door to the interior.

synthetic diets. Autoclaving used to produce sterility of diets and equipment and heat sterilization produce special problems.

After the animals are raised, they can breed and suckle their own young. The problem is lessened somewhat for those animals, such as birds, which can feed themselves from birth. Furthermore, the germ-free animals must be healthy and approach some dependable standards.

Not all of the investigated species have been raised through maturity and into other generations. This has been due partly to choice and partly to limitations in equipment and time. For example, chickens are seldom bred into successive generations because they are useful in the first generation and can be raised more easily than can mammals. On the other hand, monkeys mature so slowly that it has not been feasible to hold them for the purpose of breeding. However, these are limitations of convenience and not of technique. It is highly probable that, if necessary, all the species mentioned could be raised through generations.

The only animals which have been raised into successive generations are: chickens through two generations, rats through eight generations, Swiss mice through seven generations, and C_3H mice through six generations. This is evidence that germ-free life can be propagated and fulfill the biological criteria for the species.

While there has been little purpose in holding germ-free animals for extended periods, we have C_3H mice, 14 months; Swiss albino mice, 24 months; and Albino

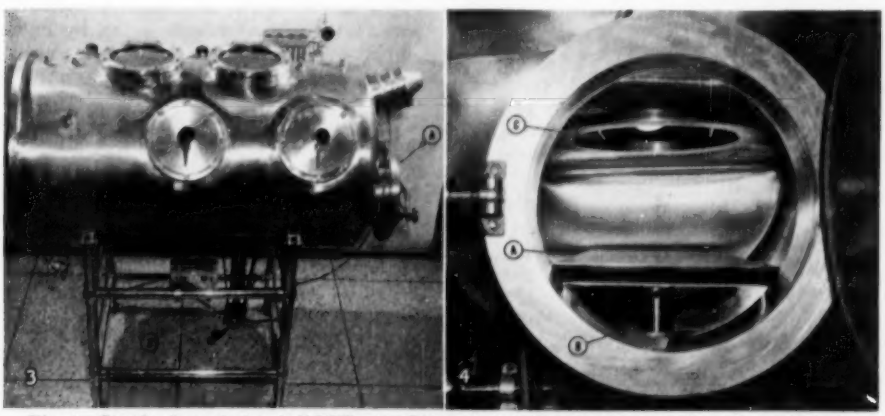


Fig. 3—Standard surgical unit ROPU-200. This unit is intended for surgical work, particularly cesarotomies. This is a two-man unit made to connect to a RSU or any of the standard units.
(A) Trap for placing pregnant animal into the germ-free unit. The opening to the unit is protected against contamination when the trap door is opened by a sheet of cellophane stretched across the opening. Surgery is performed by using a cautery and by burning through the cellophane.
(B) Unit for raising the pregnant animal into contact with the cellophane laparotomy sheet.
Fig. 4—Operating trap through which pregnant animal is introduced into ROPU-200 unit.
(A) Operating board, (B) table mechanism by which animal board is raised into contact with cellophane protective barrier, and (C) cellophane protective barrier which separates contaminated pregnant animal from sterile interior.

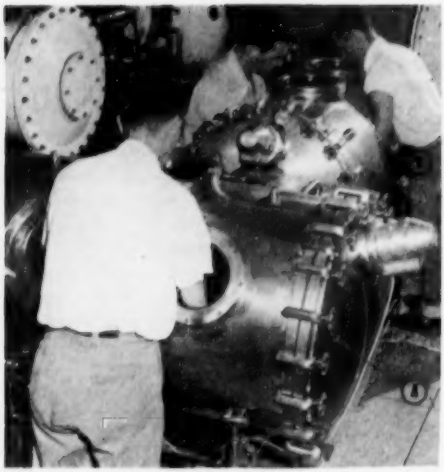


Fig. 5—Combination of germ-free units to form operating raising system.
(A) Operator in gloves of RSU-400 raising unit waiting to receive cesarean-born animals, and (B) operators in gloves of ROPU-200 surgical unit performing cesarotomy.

rats, 23 months old. The animals are healthy and active, indicating that the germ-free state does not act adversely on longevity for these species.

The earlier work with germ-free life was aimed primarily at answering the question proposed by Pasteur,[9] 1885, of whether life was possible in the absence of bacteria. Earlier experiments usually ended in contamination. The possibility of accidental contamination is by no means the problem it was. More experience with the available apparatus and improved design should still further reduce this hazard.

The number of germ-free animals it is possible to produce is merely a matter of the amount of apparatus available. The number produced at Lobund Institute over the past five years is shown (table 1). This table includes only animals raised up to the time of use in an experiment.

Thus it is possible to conclude, from experience of more than 25 years, that germ-free animals can be raised, that they will reproduce, and that the numbers are limited only by the equipment available.

3) *Apparatus Necessary to Raise and Use Germ-Free Life.*—In 1928, we raised chickens in simple churn jars until they exhausted their food and water.[10] However, if the object is to establish routines in gnotobiotics,* [23] it is necessary to standardize apparatus and to combine it into systems for the purpose. Two main systems have been devised. System 1 is intended primarily for cold sterilization with germicides either liquid or gas and will not be considered further in this report. System 2 depends on sterilization with steam under pressure and has proved by far the most satisfactory.

The Reyniers germ-free system 2 is composed of a series of units which can be fastened together into various combinations so that they will function as a system in which animals may be raised and used experimentally. While the system is intended to be used with steam sterilization, it may be used with cold sterilization, but the use of germicides is by no means certain and adaptable to routines which can be maintained by technicians. The main features of the system lie in the *in situ* sterilization of air filters and food, and in the combination of the units so that animals may be passed from one unit to another without contamination.

The standard units generally composing a complete system are: raising and holding units (RSU, fig. 1), examining units (REXU, fig. 2), and operating unit (ROPU, fig. 3 and 4). These units can be constructed of various sizes and lengths and with multiple glove ports. They are made to attach to one another by sterile locks or by end-to-end connections (fig. 5). Only with a sterile lock connection can the units be separated and maintain their sterility.

A satisfactory germ-free system can be made of the following numbers and kinds of units: 16 RSU raising and holding units, one ROPU operating unit, two REXU, and one REXU-S special examining unit. A system of this size permits ten units to be used for production of animals and ten units for experimental purposes. Smaller numbers of such units limit the use in experiments unless a central source of supply for germ-free animals is available. The cost of such a system installed with the necessary air ventilation, conditions, etc., exclusive of building, is about $100,000.

Reyniers-Trexler Germ-Free Colony Unit.—Although the standard germ-free units may be used for raising animals, they have limited use with respect to colonies. For this purpose and also for raising larger

* Greek, *gnosis*—knowledge, and *bios*—life.

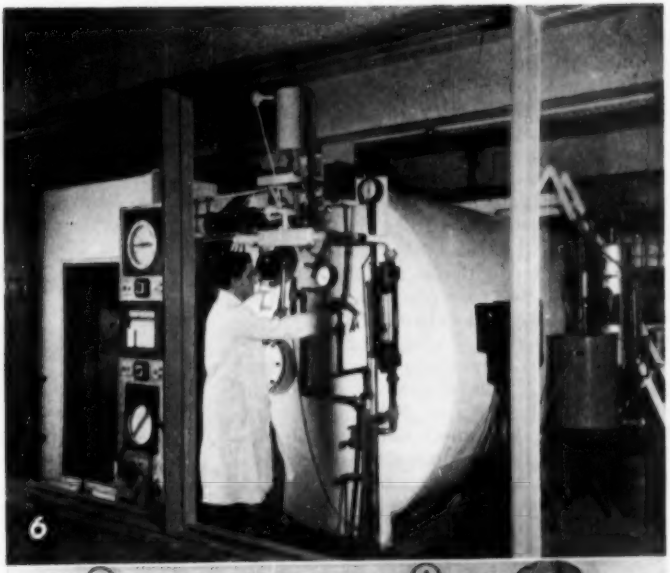


Fig. 6—Reyniers-Trexler germ-free colony unit which has been in operation at Lobund Institute since 1952.

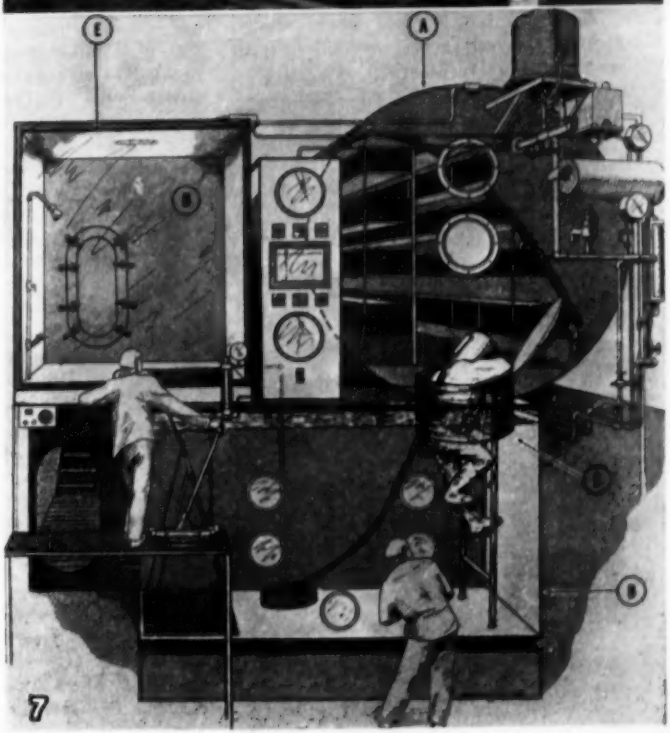


Fig. 7—Diagram of germ-free colony unit. (A) Main colony tank, (B) germicidal tank, (C) trap to main colony tank, (D) entrance to dressing rooms, and (E) germicidal shower room.

animals such as swine and goats, as well as experiments where a large number of animals are needed in a series, e.g., antibiotics and growth, a larger germ-free system has been used (fig. 6 and 7). This consists of a large tank which is 8 feet in diameter by 18 feet long, with a passage lock 20 inches in diameter. The system is sterilized with steam under pressure. Animals are moved in and out of the colony unit through the standard sterile lock. This unit is operated with the aid of an attendant who is sealed in a plastic suit supplied with air intake and exhaust (fig. 8). The suit is sterilized by a system of germicidal sprays, and by immersion into a dip tank of germicide. This system is capable of holding 1,200 rats and 300 chickens, the maximum an attendant can service in a single entry. Larger animals can be raised in the system. The colony unit is made to operate in pairs or multiples because of the danger of contamination due to germicidal sterilization. Thus, if one unit becomes contaminated, it can be sterilized and animals moved from a second unit in sufficient numbers to keep up over-all production in the colony.

Transport of Germ-Free Animals.—In order to service smaller installations of three to five standard germ-free units, a system has been devised to transport germ-free animals from a center unit to another location. This should provide wider usage

Fig. 8—Operator in germ-free colony tank.

Fig. 9—Germ-free transport units and vehicle.

of germ-free life and a more standard animal since the central colony can be genetically controlled.

The transport of germ-free animals without contamination involves three steps: (*a*) a satisfactory transport unit and vehicle, (*b*) removal of the animals from the colony, and (*c*) transfer of the animals to a germ-free system.

The basic germ-free transport unit and the transport vehicle are shown (fig. 9). A Studebaker station wagon, specially engineered by the Studebaker-Packard Corp. and equipped with temperature and humidity control and with filtered air, was designed to operate up to 1,000 miles. Tests have shown that a 2,000-mile range is not difficult.

In the past ten years, we have received more than 190 requests for the use of germ-free animals and facilities. Many originated in other countries. Only a few could be activated because of limited facilities.

A series of studies have been activated in collaboration with other scientists and institutions. Only a few examples are given at this time as illustrations of published results.

1) *Use of Germ-Free Animal Technique in the Study of Experimental Dental Caries (with the University of Chicago, Zoller Memorial Dental Clinic).*—The first phase was set up to determine the possible role of bacteria in the etiology of dental caries.[3] A diet cariogenic to conventional rats showed no lesions, microscopic or gross, in germ-free rats of the same strain. It was deduced from this evidence that dental

caries in the rat is not possible in the absence of microorganisms. The next phase [30] of the study set out to determine which microorganism(s) might be capable of producing typical carious lesions in the rat. The lactobacilli and enterococci were the organisms of choice. Germ-free rats were contaminated with pure cultures of each organism and fed cariogenic diet. Carious lesions were produced with enterococci alone in every rat. The lactobacilli produced few lesions. Other microorganisms are presently being studied and the next phase will consider nutrition.

2) *Ameba-Bacterial Relationships (with the National Institutes of Health).*—Characteristic lesions can be induced by implantation of *E. histolytica* in the cecum of the conventional guinea pig.[4] None of the germ-free guinea pigs develop amebic lesions, nor do the amebas survive for more than five days. Acute ulcerative amebiasis was produced in guinea pigs in which *Escherichia coli* and *Aerobacter aerogenes* were the monocontaminants. The results indicate that although *E. histolytica* is unquestionably the causative organism of intestinal amebiasis, the responsibility must be shared with other microorganisms. In the absence of such microbial associates, the ameba appears to be a harmless microbe incapable of independent survival in the intestine. The question now is what factors are responsible for the infection.

3) *The Synthesis of Biologically Labile Methyl Groups (with Cornell University).*—It has been shown that germ-free rats are capable of synthesizing the labile methyl group and that the degree of synthesis is comparable to that observed in conventional rats.[24]

4) *Gross Development and Vitamin-Utilization Studies in White Leghorn Chickens (with Parke, Davis and Co.).*—It was found that additional sources of vitamins did not significantly increase the growth of germ-free chickens and that large quantities of vitamins were found in the cecal contents of germ-free as well as conventional chickens, showing that vitamins found in the lower intestinal tract are not necessarily of bacterial origin.[25]

These are examples of but a few of the uses to which germ-free animals have been put. They have also been used in studies on radiation sickness,[5] hemorrhagic shock, antibiotics and growth,[26] vitamin metabolism,[27] tumorigenesis, liver necrosis,[28] and many other studies.

No mention has been made of the use of germ-free animals in such studies as host-contaminant relationships.[1, 7, 29]

DEVELOPMENT OF GNOTOBIOTICS (GERM-FREE LIFE STUDIES)

The question now is how best to make the techniques and germ-free animals more widely available to science and to individual investigators. Success depends upon how well routines can be established, capable of being carried out by technically trained people, and this, in turn, will always depend on the apparatus. The most certain way of operating such apparatus is by the use of steam under pressure in sterilizing it and in the operations necessary to raising germ-free animals. This means that such apparatus must be built strong enough to withstand the heat and pressure of steam. While it is possible to build relatively light apparatus and sterilize it in an autoclave, the actual saving in cost of apparatus and operation is not great.

There are two ways in which the use of germ-free life can be extended: (*a*) simpler apparatus and (*b*) the establishment of relatively small installations (3 to 5 germ-free units), capable of being steam-sterilized, in which experiments can be conducted, and to which germ-free animals raised at a larger center can be shipped.

It is possible to raise animals such as chickens in a very simple apparatus (fig. 10), such as the churn jars, sterilized in an autoclave. In this type of apparatus, chickens can be hatched and held for periods up to 30 days, or as long as the ration and water last. They can be examined only at the end of the experiment but modifications of this type of apparatus can

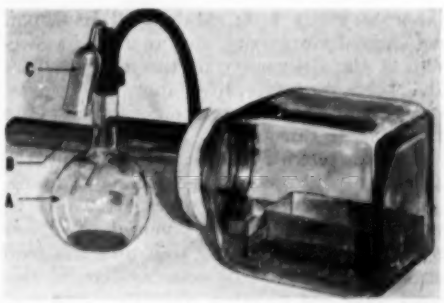


Fig. 10—Jar-type unit for raising germ-free chickens. (A) Water flask, (B) air inlet and outlet filters, and (C) filter on water flask.

be made. Simple apparatus sterilized with chemicals to which rubber gloves are attached can also provide a less expensive system.[20] In this type of apparatus it is possible to raise small mammals through the suckling period until the failure of the apparatus in sterility. Sterile hoods also have limited use,[19] and these can be sterilized with chemicals or ultraviolet light. Also the apparatus of Gustafsson[17] can be employed and this is sterilized in an autoclave. In this apparatus, it is possible also to do excellent work but it is not simpler or less expensive to construct in this country. Many other simple apparatus can be devised and will be improved. The point is that there is no reason why someone interested in germ-free life can not get started in the field.

The other line of development involves the establishment of several centers large enough to raise colonies of germ-free animals and to provide facilities for investigators to work. The limitations lie in the difficulty for investigators to leave their own laboratories for extended periods of time. The advantages, however, are in the source of animals and in the experience of technicians in operating the germ-free equipment. On a basis of our long experience in the field, we favor this plan and see in it a fuller development of the use of germ-free animals in experimental biology and medicine.

The first full-scale development of gnotobiotics was at Lobund Institute and until recently it was the only source in the world for such work. There is now a laboratory and a 20-unit system in operation at Walter Reed Army Medical Center, Washington, D. C. Another has been established but is just going into operation at National Institutes of Health, Bethesda, Md. This is a 15-unit system. One such laboratory has been established in Japan at Nagoya University and another in Sweden at the University of Lund. These are the only germ-free life laboratories presently in existence, but others are being planned in Europe.

In this country it is well within the means of the federal government to organize germ-free life centers. It would be curious, considering the amount of money spent each year in research and the lead in this field that this country has, if this lead were lost to other countries.

One thing is certain, there is no lack of problems and no lack of need for such facilities. It is certain that the need will increase.

References

[1] Reyniers, J. A., and Trexler, P. C.: Germfree Research: A Basic Study in the Host-Contaminant Relationship. I. General and Theoretical Aspects of the Problem. Bull. New York Acad. Med., *31*, (1955): 231–242.

[2] Reyniers, J. A.: The Significance of Germfree Life Methodology (Gnotobiotics) of Experimental Biology and Medicine. M.S.C. Vet., *13*, (1953): 178–185.

[3] Orland, F. J., Blayney, J. R., Harrison, R. W., Reyniers, J. A., Trexler, P. C., Wagner, M., Gordon, H. A., and Luckey, T. D.: Use of the Germfree Animal Technic in the Study of Experimental Dental Caries. J. Dent. Res., *33*, (1954): 147–174.

[4] Phillips, B. P., Wolfe, P. A., Rees, C. W., Gordon, H. A., Wright, W. H., and Reyniers, J. A.: Studies on the Amoeba-Bacteria Relationship in Amoebiasis—Comparative Results of the Intracecal Inoculation of Germfree, Monocontaminated, and Conventional Guinea Pigs with *Endamoeba Histolytica*. Am. J. Trop. Med. and Hyg., *4*, (1955): 675–692.

[5] Reyniers, J. A., Trexler, P. C., Scruggs, W., Wagner, M., and Gordan, H. A.: Observations on Germfree and Conventional Albino Rats After Total-Body X-Radiation. Abst. Radiation Res., *5*, 1956.

[6] Reyniers, J. A.: The Control of Contamination in Colonies of Laboratory Animals by the Use of Germfree Techniques. Anim. Care Panel (in press).

[7] Gordon, H. A.: Germfree Research: A Basic Study in Host-Contaminant Relationship. III. Morphologic Characterization of Germfree Life. Bull. New York Acad. Med., *31*, (1955): 239–242.

[8] Reyniers, J. A.: Germfree Life Methodology (Gnotobiotics) and Experimental Nutrition. Proc. Third Internat. Cong. Biochem., Brussels, Belgium, Academic Press, Inc., 1956.

[9] Pasteur, L.: Observations relatives à la note . . . de M. Duclaux. Compt. rend. Acad. sci., *100*, (1885): 66–68.

[10] Nuttal, G. H. F., and Thierfelder, H.: Thierisches Leben ohne Bakterien im Verdauungskanal. Hoppe Seyler's Ztschr. f. physiol. Chem., *21*, (1895–1896): 109–121.

[11] Schottelius, M.: Die Bedeutung der Darmbacterien für die Ernährung. Arch. f. Hyg., *34*, (1899): 210–243.

[12] Cohendy, M.: Expériences sur la vie sans microbes. Ann. Inst. Pasteur, *26*, (1912): 106–137.

[13] Kuster, E.: Die Gewinnung, Haltung und Aufzucht keimfreier Tiere und ihre Bedeutung für die Erforschung natürlicher Lebensvorgänge. Arb. a. d. k. Gsndhtsamt., *48*, (1915): 1–79.

[14] Reyniers, J. A.: The Use of Germ-Free Guinea Pigs in Bacteriology. Proc. Ind. Acad. Sci., *42*, (1932): 35–37.

[15] Glimstedt, G.: Bakterienfreie Meerschweinchen. Aufzucht, Lebensfähigkeit und Wachstum, nebst Untersuchungen über das Lymphatische Gewebe. Acta path. et microbiol. Scand., suppl. *30*, (1936): 1–295.

[16] Balzam, N.: Élevage aseptique des animaux. I. Appareillage et méthode. II. Élevage aseptique des poules maintenues aux régimes complets et déficients. Ann. de physiol., *13*, (1937): 370–385.

[17] Gustafsson, B.: Germ-Free Rearing of Rats. Acta path. et microbiol. Scand., suppl. *73*, (1948): 26–43.

[18] Reyniers, J. A., and Trexler, P. C.: The Germ-Free Technique and Its Application to Rearing Animals Free from Contamination. Micrurgical and Germ-Free Methods, Chas. C. Thomas, Springfield, Ill. (1943): 114–143.

[19] Lobund Institute: Lobund Report No. 1. University of Notre Dame Press, Notre Dame, Ind., (1946): 7–9.

[20] Lobund Institute: Lobund Report No. 2. University of Notre Dame Press, Notre Dame, Ind. (1949): 38.

[21] Lobund Institute: Lobund Report No. 3. University of Notre Dame Press, Notre Dame, Ind. (in press).

[22] Cohendy, M., and Wollman, E.: Expérience sur la vie sans microbes. Élevage aseptique de cobayes. Compt. rend. Acad. sci., *158*, (1914): 1283–1284.

[23] Reyniers, J. A., Trexler, P. C., Ervin, R. F., Wagner, M., Luckey, T. D., and Gordon, H. A.: The Need for a Unified Terminology in Germ-Free Life Studies. Lobund Report No. 2, University of Notre Dame Press (1949): 151–162.

[24] DuVigneaud, V., Ressler, C., Rachel, J. R., Reyniers, J. A., and Luckey, T. D.: The Synthesis of "Biologically Labile" Methyl Groups in the Germ-Free Rat. J. Nutr., *45*, (1951): 361–376.

[25] Reyniers, J. A., Trexler, P. C., Ervin, R. F., Wagner, M., Luckey, T. D., Gordon, H. A., Brown, R. A., Mannering, G. J., and Campbell, C. J.: Germ-Free Chicken Nutrition. I. Gross Development and Vitamin Utilization Studies Employing White Leghorn Chickens. J. Nutr., *41*, (1950): 31–49.

[26] Reyniers, J. A., Luckey, T. D., and Gordon, H. A.: Studies on the Growth Effect of Antibodies in Germfree Animals. A colloquium, June 4, 1952.

[27] Luckey, T. D., Pleasants, J. R., and Reyniers, J. A.: Germfree Chicken Nutrition. II. Vitamin Interrelationships. J. Nutr., *55*, (1955): 105–118.

[28] Luckey, T. D., Reyniers, J. A., Gyorgy, P., and Forbes, M.: Germfree Animals and Liver Necrosis. Ann. New York Acad. Sci., *57*, (1954): 932–935.

[29] Wagner, M.: Germfree Research: A Basic Study in Host-Contaminant Relationship. II. Serologic Observations in Germfree Animals. Bull. New York Acad. Med., *31*, (1955): 236–239.

[30] Orland, F. J., Blayney, J. R., Harrison, R. W., Reyniers, J. A., Trexler, P. C., Ervin, R. F., Gordon, H. A., and Wagner, M.: Experimental Caries in Germfree Rats Inoculated with Enterococci. J. Am. Dent. A., *50*, (1955): 259–272.

A Simple Ear Punch for Use on Laboratory Rats

BENNETT J. COHEN, D.V.M., Ph.D., and CHESTER CHALBERG

Los Angeles, California

EAR NOTCHING and punching methods have long been used for the permanent identification of laboratory rats and mice.[1,2] Poultry toe clips, leather and ticket punches, and surgical scissors are some of the instruments which have been used. However, in this laboratory, the poultry clip has not provided adequate leverage for punching ears of large rats, the leather and ticket punches have not permitted easy visualization of the ear for precise localization of the punching site, and the scissors are useful for notching only. These disadvantages have been overcome by the design of a simple inexpensive tool which can be made in any institutional shop. Using this tool, as many as four holes or notches can be made in each ear, and many hundreds of rats can be numbered serially.

A snipe-nosed plier, made of tempered steel, is used (fig. 1 and 2). The pliers are annealed and small holes are drilled to receive a shearing pin made from the stock end of a dental drill (fig. 1 and 2, A). The pin dimensions are 0.09 inches in diameter by 0.25 inches long, with the shearing hole providing only minimal clearance to insure proper cutting action (fig. 1 and 2, B). The pliers are retempered after the holes are drilled, and the pin is fixed in place with silver solder. A small steel stop is similarly fixed to prevent bruising of the ears as the pliers are closed during the punching procedure (fig. 1 and 2, C). Springlike action is provided by means of a short piece of spring steel bolted to one handle as shown (fig. 1 and 2, D).

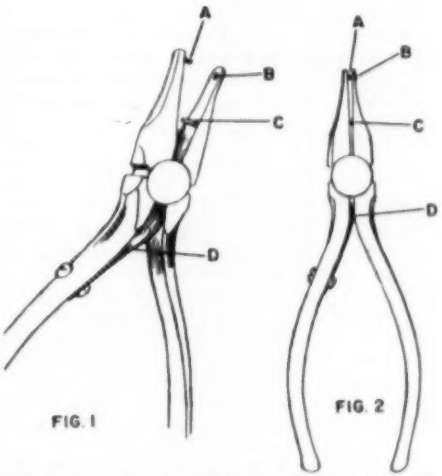


Fig. 1 and 2—Instrument for punching ears of rats.

Presumably, the same instrument can be used for mice. However, a slightly smaller shearing pin should probably be inserted if the punch is to be used only for this species.

From the Office of Animal Care and the Departments of Physiology and Biophysics, University of California Medical Center, Los Angeles.

References

[1] Farris, E. J., and Griffith, J. Q. (ed.): The Rat in Laboratory Investigation, 2nd ed. J. B. Lippincott Co., Philadelphia (1949): 16.

[2] Snell, G. D. (ed.): Biology of the Laboratory Mouse. Dover Publications, Inc., New York (1941): 477.

Heat-Inactivated Vesicular Stomatitis Virus as Antigen in the Complement-Fixation Test

G. T. DIMOPOULLOS, Ph.D.; O. N. FELLOWES, Ph.D.; J. J. CALLIS, D.V.M., M.S.; G. C. POPPENSIEK, V.M.D., M.S.; J. TESSLER, M.S.; W. R. HESS, Ph.D.

Greenport, Long Island, New York

WHENEVER POSSIBLE an inactivated virus antigen is preferable in serological and other laboratory tests, especially when such virus in the active state is transmissible to man and a variety of animals.[1,2]

Shahan [3] reported that vesicular stomatitis virus (VSV) in guinea pig foot pads was rendered noninfective for guinea pigs when heated at 55 or 60 C. for 30 minutes. On the other hand, Madin [4] reported that VSV in allantoic fluid, after exposure to 56 C. for 30 or 60 minutes, killed chicken embryos and mice; the embryos and mice presumably died of VSV.

This study was designed to determine (1) the heat required to render VSV noninfective and (2) the feasibility of use of such heat-treated material as antigen in the complement-fixation (CF) test.

MATERIALS AND METHODS

PREPARATION OF ANTIGENS

Both New Jersey [5] and Indiana [6] types of VSV were used. Infected bovine tongue epithelium was ground in a mortar and suspended, 1 part in 9 parts of tryptose phosphate broth at pH 7.4. The virus suspension was centrifuged at 2,000 r.p.m. for 20 minutes, and some of the supernatant fluid was placed in each of two 30-ml., flat-bottom vials (25 by 90 mm.). Each vial contained a small, plastic-covered magnet and was fitted with a one-hole rubber stopper equipped with a precision thermometer. This assembly was placed in a water bath (accurate to ± 0.01 C.), resting on a large magnetic stirrer. With this arrangement, the suspension was stirred during heating (fig. 1). The 30-minute heating period was recorded after the material in the vials reached the desired temperatures of 56 or 60 C. The lag in time necessary to bring the contents of the vials to 56 or 60 C. was approximately three minutes.

After heating, the samples were immediately chilled. Penicillin and streptomycin were added in the amounts of 1,000 units and 1 mg., respectively, per milliliter of suspension and held at 4 C. for one hour. The virus suspensions were then diluted in physiological saline for the complement-fixation (CF) tests and in tryptose phosphate broth for the infectivity tests in suckling mice (7 to 9 days old), cattle, guinea pigs, and embryonating chicken eggs. In the infectivity tests of bovine kidney tissue culture, tissue-culture diluting fluid was used in preparing the dilutions.[7] The heating and stirring assembly was not available during the work with the Indiana-type virus. Therefore, suspensions used in these experiments were not agitated during heating in a serological water bath.

INFECTIVITY TESTS

Dilutions of 10^{-1} through 10^{-8} of the heated suspensions were used in all trials. These suspensions were diluted to avoid complication due to the interference phenomenon, which may be exhibited by concentrated suspensions that have been heat treated.[8] In the unheated samples, dilutions appropriate for obtaining 50 per cent end points were used.

Embryonating Chicken Eggs.—Dilutions of the heated and unheated control suspensions of VSV were inoculated in 0.2-ml. amounts into the allantoic cavity of 7- to 8-day-old embryonating chicken eggs, using 10 eggs per dilution. After inoculation, the eggs were incubated at 35 C. and candled daily for 12 days. Embryo mortality within the first 24 hours postinoculation was considered to be due to nonspecific causes and was not included in the final computation. Embryos which died after 24 hours' incubation following inoculation with the heated material were examined, and the chorioallantoic membranes (c.a.m.) harvested. The c.a.m.

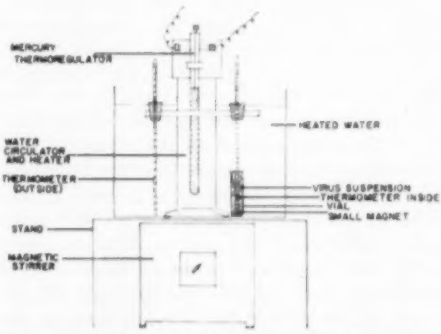


Fig. 1—Heating and mixing assembly for suspensions of vesicular stomatitis virus in sealed vials.

From Plum Island Animal Disease Laboratory, Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Department of Agriculture, Greenport, Long Island, N. Y.

The present address of Dr. Dimopoullos is Department of Veterinary Science, Louisiana State University, Baton Rouge.

were ground in a mortar, diluted to a 10 per cent suspension, treated with antibiotics, and inoculated into 5 additional embryonating chicken eggs for as many as six serial passages. These suspensions were tested as antigens in the CF test. The c.a.m. at each passage were also harvested and used as antigens in the CF test.

Suckling Mice.—Dilut .as of the control and heated suspensions of VSV were inoculated intraperitoneally in 0.05-ml. amounts into 7- to 9-day-old mice, using 10 mice per dilution. The mice were observed for paralysis and death for 14 days after inoculation. Any mice inoculated with heated virus suspensions which died later than 24 hours postinoculation were necropsied, and the brain and skeletal muscle harvested. The tissues were ground, diluted to a 10 per cent suspension, and treated with antibiotics. These preparations were inoculated into mice and embryonating chicken eggs for as many as six serial passages. Each harvest of brain and muscle was tested for the presence or absence of VSV, using the CF test.

Guinea Pigs.—Dilutions of the heated and control VSV suspensions were inoculated intradermally, 0.1-ml. amounts into each metatarsal pad, using 10 guinea pigs per dilution. The animals were observed for vesiculation of the pads for a period of eight days. At the end of this time, each animal not responding to primary inoculation was challenged with a 10 per cent suspension of active guinea pig-adapted VSV.

Cattle (Grade Hereford Steers, 18 to 36 Months Old).—Dilutions of the heated and control suspensions of VSV were inoculated intradermalingually, 0.1 ml. per site, into five sites for each dilution, using four dilutions per tongue and 2 animals per sample.[9] The animals were observed daily for vesiculation and other changes for eight days or longer, after which time those not responding to the inoculations were challenged with a 10 per cent suspension of active bovine-adapted VSV.

Tissue Culture.—Trypsin-dispersed calf kidney cells which had been propagated in stationary tubes for seven days were inoculated with dilutions of the heated and unheated VSV suspensions, using 0.4 ml. per tube and ten tubes per dilution. Each tube contained 0.8 ml. of a medium composed of 0.5 per cent lactalbumin hydrolyzate and 2 per cent bovine serum in Hanks' solution. The tubes were observed daily for three days for the cytopathogenic effect produced by VSV.[7]

The 50 per cent end-point method of Reed and Muench[10] was used to determine the $l.d._{50}$ and $i.d._{50}$ values in all infectivity experiments.

COMPLEMENT FIXATION

The technique of the CF test was essentially that described by Rice and McKercher.[11] The heated and control suspensions of VSV were diluted twofold in physiological saline and used in 0.25-ml. quantities. Anti-VSV guinea pig serum in 0.25-ml. quantity was added to each tube. The mixtures were shaken, and 0.25 ml. of normal guinea pig serum containing 1.6 units of complement was added. The tubes were then shaken and placed in a water bath at 37 C. for 20 minutes. The hemolytic indicator system of 0.5 ml. of sensitized sheep red blood cells (0.25-ml. cells and 0.25-ml. amboceptor containing 4 units) was added to each tube. The cell suspension had a hemolyzed optical density of 0.5 when measured at a wave length of 580 mμ. The tubes were shaken, placed in a water bath at 37 C. for 30 minutes, and read for the presence or absence of hemolysis. Complete absence of hemolysis was designated as 4 (strongly positive), increasing to various degrees of hemolysis—3 (moderately positive), 2 (weakly positive), 1 (trace), and complete hemolysis (negative). Proper serum, antigen, complement, hemolytic, and cell controls were included in the tests.

TABLE 1—Infectivity of Bovine Tongue Epithelial Suspensions Containing New Jersey-Type Vesicular Stomatitis Virus Heated at 56 or 60 C. for 30 Minutes and Inoculated into Various Host Mediums

Medium inoculated	Infectivity of heated suspensions	$l.d._{50}$/ml. or $i.d._{50}$/ml. of unheated virus control
Embryonating chicken eggs	—	8.2
Unweaned mice	—	8.8
Guinea pigs	—	5.5
Cattle	—	6.9
Tissue cultures	—	4.5*

* The $i.d._{50}$/ml. of VSV in cultures of calf kidney cells in these trials was lower than previously reported titers of VSV using guinea pig kidney cell cultures as the test medium.[7]

RESULTS

Infectivity Determinations.—Representative experiments in various mediums for determining the effect of heat on the infectivity of New Jersey type of VSV are shown (table 1). Under the conditions described, temperatures of 56 or 60 C. for 30 minutes rendered tissue suspensions of New Jersey-type VSV noninfectious for suckling mice, cattle, guinea pigs, embryonating chicken eggs, and tissue cultures of calf kidney cells. The cattle and guinea pigs which remained negative were challenged with active virus and were shown to be fully susceptible to VSV.

Indiana-type VSV, which was tested in mice and embryonating chicken eggs, was also found noninfectious. As previously stated, this part of the work differed only in the type of water bath used.

In some cases, chicken embryos and mice died after inoculation with heated virus suspensions. The c.a.m. from such embryos and the brains or skeletal muscles from the

TABLE 2—Complement-Fixing Activity of Heated and Control Suspensions of Bovine Tongue Epithelium Infected with New Jersey-Type Vesicular Stomatitis Virus

Treatment	Dilutions of virus suspension							Antigen control
	Undil.	1/2	1/4	1/8	1/16	1/32	1/64	
Unheated control	4	4	4	4	4	2	—	—
56 C. for 30 minutes	4	4	4	4	—	—	—	—
60 C. for 30 minutes	4	4	4	4	4	—	—	—

mice were harvested, ground, diluted, and passed into eggs and mice for six serial passages. Suspensions of tissue from each passage were subjected to the CF test, and in no case was the presence of VSV indicated.

Complement-Fixation Tests.—A representative experiment to determine the effect of heat on the antigenicity of the New Jersey type of VSV is given (table 2). Heating under the conditions described affected the complement-fixing activity to a slight degree. There was only a twofold decrease in final titer of heated versus control VSV suspensions. Heated virus suspensions were found to have adequate complement-fixing activity when used as antigens in the CF test. No anticomplementary and hemolytic properties or objectionable turbidity were observed.

Essentially the same results were obtained with active and heat-inactivated antigens of the Indiana type of VSV.

SUMMARY AND CONCLUSIONS

1) Suspensions of bovine tongue epithelium containing New Jersey or Indiana types of vesicular stomatitis virus (VSV) were subjected to heating at 56 or 60 C. for 30 minutes.

2) Dilutions of control and heated suspensions of New Jersey-type VSV were inoculated into suckling mice, cattle, guinea pigs, embryonating chicken eggs, and tissue cultures of calf kidney cells. In experiments with the Indiana-type VSV, only mice and embryonating chicken eggs were used.

3) Heating of tissue suspensions containing VSV rendered them noninfective for the mediums used, whereas the complement-fixing activity of heated VSV suspensions was not significantly affected.

4) The heated suspensions did not exhibit objectionable turbidity or hemolytic and anticomplementary properties.

5) Vesicular stomatitis virus in bovine tongue epithelial suspensions, which have been heated as indicated, may be used as antigen in the complement-fixation test.

References

[1] Hanson, R. P., Rasmussen, A. F., Brandly, C. A., and Brown, J. W.: Human Infection with the Virus of Vesicular Stomatitis. J. Lab. Clin. Med., *36*, (1950): 754–758.

[2] Fellowes, O. N., Dimopoullos, G. T., and Callis, J. J.: Isolation of Vesicular Stomatitis Virus from an Infected Laboratory Worker. Am. J. Vet. Res., *16*, (1955): 623–626.

[3] Shahan, M. S.: Effect of Temperature, Phenol, and Crystal Violet on Vesicular Stomatitis Virus. Am. J. Vet. Res., *7*, (1946): 27–31.

[4] Madin, S. H.: The Effect of Temperature on the Infectivity of Vesicular Stomatitis Virus, Sixth Status Report. Vol. II, Sect. IV, ONR Contract, N7-29563 with University of California (Jan. 1–March 3, 1952): 2–3.

[5] Mott, L. O., U. S. Department of Agriculture, Agricultural Research Service, Animal Disease and Parasite Research Branch, Beltsville, Md.: Personal communication, 1956.

[6] Heiny, E.: Vesicular Stomatitis in Cattle and Horses in Colorado. North Am. Vet., *26*, (1945): 726–730.

[7] Bachrach, H. L., Hess, W. R., and Callis, J. J.: The Growth and Cytopathogenicity of Vesicular Stomatitis Virus in Tissue Culture. J. Immunol., *73*, (1955): 186–191.

[8] Henle, W.: Interference Phenomena Between Animal Viruses: A Review. J. Immunol., *64*, (1950): 203–236.

[9] Henderson, W. M.: The Quantitative Study of Foot-and-Mouth Disease Virus. Agricultural Research Council Report Series No. 8, HMS Office, London (1952): 16–24.

[10] Reed, L. J., and Muench, H.: A Simple Method of Estimating Fifty Percent End Points. Am. J. Hyg., *27*, (1938): 493–497.

[11] Rice, C. E., and McKercher, P. D.: Studies of the Complement-Fixation Reaction in Virus Systems. VI. In Vesicular Stomatitis in Horses, Cattle and Swine. J. Immunol., *73*, (1954): 309–317.

A Simple Technique for the Inoculation of the Chorioallantoic Membrane of Chicken Embryos

JOHN R. GORHAM, D.V.M., Ph.D.

Pullman, Washington

MANY METHODS have been reported for the inoculation of the chorioallantoic membrane (c.a.m.) of incubating hens' eggs. In general, they are variations of Burnet's "dropped membrane" technique.[1] The method described herein is a modification of the air sac approach first reported by Brandly.[2]

In Brandly's method, a triangle or circle is cut in the shell and outer shell membrane and the test material inoculated beneath the inner shell membrane onto the c.a.m. A slight elevation and wetting of the inner shell membrane at the site of inoculation is the criterion of satisfactory inoculation.

It was accidentally discovered in this laboratory that if the inoculum is dropped onto the inner shell membrane and if the inner shell membrane and the c.a.m. are pierced with the needle, the inoculum will pass rapidly onto the c.a.m. Some virus is also carried into the allantoic sac. Thus, the need for visualization of the inner shell membrane is eliminated.

Insertion of the needle through a small hole over the center of the air sac and release of the inoculum in the air sac was suggested by Gillespie.[3] This technique has now been used in commercial production and state and federal laboratories for the cultivation of a number of different viruses. Fabricant [4] reported that this technique is satisfactory for the isolation of fowlpox, infectious laryngotracheitis, Newcastle disease, and infectious bronchitis viruses. Furthermore, the method is more satisfactory for the initial isolation of the infectious bronchitis virus than the allantoic route.

The method for the growth of modified distemper and bluetongue virus in chicken embryos, as used in this laboratory, is as follows: After eight days' incubation, the eggs are examined by transillumination to determine the viability of the embryo. The shell over the air sac is painted with an alcoholic solution of iodine. An opening, sufficiently large to admit a needle, is made with a nail point or drill in the center of the blunt end of the egg (fig. 1). A tuberculin syringe and a 1¼- to 1½-inch, 21- to 24-gauge needle are employed. With the egg in a vertical position, the needle is inserted through the hole to a depth of about ¼ inch, and 0.2-ml. of inoculum is released into the air sac. The needle is passed down the long axis of the egg to a depth of about 1 inch and removed. The opening in the shell may be closed with a drop of collodion or melted paraffin. The inoculated embryos are incubated in trays in an incubator having an automatic turning device.*

After incubation, the egg is moistened with antiseptic solution and a hole is cut at the pointed end of the egg with a pair of scissors. This hole should be just large enough to release the embryo by gentle

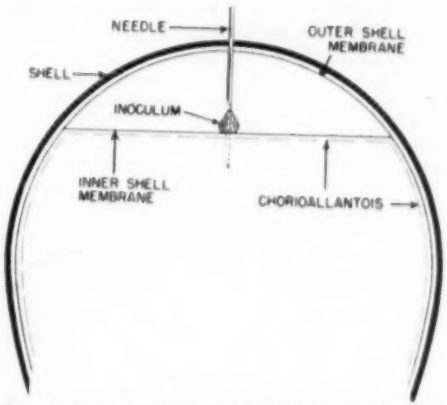


Fig. 1—Insert needle vertically into the air sac end of the egg and deposit inoculum onto the inner shell membrane. Continue insertion along the long axis of the egg, puncturing the inner shell membrane and the chorioallantois.

From the Animal Disease and Parasite Research Division, Agricultural Research Service, U. S. Department of Agriculture, and the Washington Agricultural Experiment Station, State College of Washington, Pullman.

Experiment station project 3173–1268–41.

* Model 252, James Manufacturing Co., Fort Atkinson, Wis.

shaking. The c.a.m. will remain adherent to the inside of the shell and can be harvested separately.

This technique can be employed for the initial isolation of various unadapted viruses to growth in chicken embryos. It permits, in a single injection, a combination inoculation of the c.a.m., allantoic chamber, and yolk chamber.

References

[1] Burnet, F. M.: A Virus Disease of the Canary of the Fowlpox Group. J. Path. and Bact., *37*, (1933): 107–122.

[2] Brandly, C. A.: Some Studies of Infectious Laryngotracheitis. J. Infect. Dis., *57*, (1935): 102–107..

[3] Gillespie, J. H.: Personal communication, 1955.

[4] Fabricant, J.: A Modified Chick Embryo Inoculation Technique for the Isolation of Viruses from the Respiratory Tract of Chickens. (Paper presented June, 1956, to the 28th annual meeting of the N.E. Pullorum Conference, Newark, Del.)

Effect of Choline Deficiency on Chronic Selenium Poisoning of Rats

IRENE ROSENFELD, Ph.D., and H. F. EPPSON, M.Sc.

Laramie, Wyoming

CONSIDERABLE LOSSES in livestock in the western states are due to acute or chronic selenium poisoning. Previous investigators have demonstrated that the toxicity of selenium was influenced by various dietary factors. Lewis *et al.*[1] reported that 30 per cent casein afforded significantly more protection against selenium intoxication with 20 to 50 p.p.m. than did an equicaloric diet of 6 per cent casein. Methionine (0.45 to 0.89%) added to a 6 per cent casein diet gave the same protection as 30 per cent casein. Rosenfeld and Beath[2] reported that a high protein diet reduced the severity of selenium toxicity.

Jacobi and Baumann[3] fed 17.5 and 70.0 p.p.m. of sodium selenite in an indirect attempt to lower the labile methyl supply in choline-deficient animals, since in selenium toxicity there is a garlic-like odor which was ascribed to the formation of methyl selenide. They reported that diets containing the above amounts of sodium selenite did not reduce the choline content of the tissues. Fels and Cheldelin[4] were able to reverse the inhibition produced by selenate in yeast culture with the addition of 0.5 to 3.0 mg. DL-methionine. Choline, betaine, homocysteine, and glutathione, either in combination or singly, were ineffective. Klug *et al.*[5] reported that methionine from 0.5 to 2.0 per cent gave no protection against selenosis. Halverson *et al.*[6] extracted a fraction from linseed oil meal with 50 per cent hot aqueous alcohol which reduced the toxicity of selenium.

In chronic selenosis, there is extensive liver damage[7] and decrease in growth rate.[8] Choline has lipotropic action and is essential for the normal growth of animals; therefore, it was thought to be of considerable value to study the effect of choline deficiency and choline supplementation on chronic selenosis.

From the Agricultural Research Chemistry Department, University of Wyoming (Rosenfeld, research pharmacologist, and Eppson, associate research chemist).

Approved for publication by the director of the Experiment Station, journal series 84.

This research was partially supported by AEC contract AT(11-1)253.

METHODS

Male and female Sprague-Dawley rats weighing 45 to 50 Gm. were used as experimental animals. The animals were allotted to groups of 5 males and 5 females each, balanced on the basis of body weight. They were fed *ad libitum*, and each animal was weighed weekly during the experimental period of 30 days. The daily food intake was recorded.

The following diets were used: choline-deficient and ground laboratory chow with or without choline. The percentage composition of the deficient diet was as follows: sucrose, 56.0; casein, vitamin-free, 20.0; vegetable oil, 20.0; and salt mixture, 4.0 (Osborne and Mendel). Vitamins were added to provide in milligrams per kilogram of ration as follows: vitamin A, 40.0 (200,000 units/Gm.); vitamin D, 2.5 (400,000 units/Gm.); *alpha* tocopherol, 50.0; niacin, 45.0; riboflavin, 10.0; pyridoxine hydrochloride, 10.0; thiamine hydrochloride, 10.0; calcium pantothenate, 30.0; biotin, 0.1; folic acid, 0.9; vitamin B_{12}, 0.02.* Chemical analysis indicated that the choline-deficient diet contained 15.0 per cent protein on a dry-weight, fat-free basis. Preliminary experiments showed that severe growth inhibition did not follow the use of choline-deficient diet. The degree of fatty metamorphosis in the liver was not extensive enough to overshadow the damage produced by selenium. When choline chloride was incorporated in the diets, it was always at a level of 0.2 per cent. The principals were given daily intraperitoneal injections of 1.5 mg. of selenium, as sodium selenate, per kilogram of body weight. The controls received the same diet but no selenium.

Necropsies were performed at the termination of the experiment (30 days) or at the time of death and samples of liver, kidney, adrenals, gonads, spleen, and pancreas were fixed in 4 per cent isotonic formalin. Sections were prepared by the usual histological techniques and stained with hematoxylin-eosin. Fat determination in the liver was carried out according to the method of Bixby *et al.*[9]

RESULTS

The growth rates of the animals on the various diets with and without selenium injection and dietary choline supplements are indicated (fig. 1). Regardless of diets used, the growth rate of the selenium-injected rats (principals) was less than that

* Purchased from the Nutritional Biochemical Corp.

of the controls. Selenium-injected animals had an average weekly gain of 11.1 Gm. less than the animals which received no selenium. The standard error of this difference was ± 1.2 gr. Indicating that the

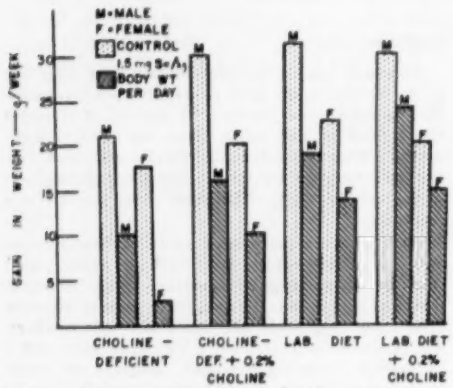


Fig. 1—Effect of choline on selenium intoxication in rats.

difference in the growth rate between selenium and nonselenium groups was highly significant. Typical signs of selenium intoxication appeared less than one week after the animals were on the choline-deficient diet plus selenium. The females in this group were far more susceptible to selenium intoxication than the males. The growth was slow and decidedly subnormal, the animals were greasy in appearance, and the fur was ruffled (fig. 2). It has been reported by previous investigators that the females are more susceptible to selenium poisoning than the males.[9] This susceptibility was more evident when the diet was deficient in choline than when the choline supplement was added to the same diet. The growth rates in the selenium-injected animals increased from an average of 6.5 Gm. to 13.5 Gm. when choline was added, suggesting that choline supplementation under our experimental conditions had beneficial effect on the growth retardation produced by selenium.

The differences in growth rates in the selenium-injected animals were partially due to differences in food intake especially in the latter part of the experiment. During the first week, both the principal and control rats consumed 10.0 to 12.0 Gm. of food per day. As the selenium injections continued, the food intake of the principals decreased to 6.0 to 7.0 Gm. per day: this effect was most prominent in rats fed the choline-deficient diet. There was some reduction in food intake as soon as selenium intoxication became evident. Animals which died of selenium intoxication usually developed anorexia one or two days before death.

The effect of choline and selenium on fat deposition in liver is shown (table 1). The level of liver fat showed some increase on the choline-deficient diet. Nino-Herrera *et al.*[10] reported higher liver fat values for animals fed choline-deficient diets containing 9.0 to 18.0 per cent casein, respectively. The choline-deficient diet used apparently contained a considerably higher concentration of labile methyl donors than did the diet used by the above investigators. The lower fat values in the choline-deficient plus selenium group were attributed to the fact that 70 per cent of the animals died within 23 days. Animals of this group which were killed at the end of 30 days had on the average 23.1 per cent fat in the liver (dry-weight basis). These results indicate that administration of selenium for 30 days had no influence on fat metabolism in the liver.

There was a definite correlation between growth rate and the percentage of survival of the principals. Animals given the choline-deficient diet plus selenium showed the least increase in body weight; only 30.0 per cent survived the 30-day experimental

Fig. 2—Female rat on left on choline-deficient diet. Female on right on choline-deficient diet plus selenium showing stunted growth and ruffled greasy fur. Animals had been on the diet for 21 days.

period. In the group receiving selenium, the addition of choline to the choline-deficient diet increased the survival rate to 70.0 per cent. When animals were fed a well-balanced laboratory diet plus choline plus selenium, 100 per cent survived the 30-day experimental period.

Histological Studies.—The discussion will be limited to the liver and kidneys, since other organs showed only slight group differences.

In choline deficiency, fatty metamorphosis in the livers was irregular in its distribution. The affected cells were enlarged and spherical. The nuclei as a rule were pushed to the periphery. The cytoplasm and nuclei of nonfatty cells showed increased hyperchromatosis (fig. 3A). No necrosis was present. Koch-Weser *et al.*[11] and Nino-Herrera *et al.*[10] reported a more diffuse fatty metamorphosis in animals fed a choline-deficient diet. However, the choline-deficient diet they used was lower in protein than the one used in our experiments. The livers of animals, which were on the choline-deficient diet and were injected with selenium and died at the early part of the experiment, showed mainly hepatic cell damage. The cytoplasm of some of the hepatic cells was swollen and vacuolated and the cell boundaries were undefined. Some of the nuclei were pyknotic while others were hyperplastic with considerable karyorrhexis. There were areas of focal necrosis but necrosis was not the dominating pathological change in this group. The sinusoids showed some dilatation (fig. 3B). Liver tissue of animals on the same diet as the above group but killed at the termination of the experiment showed localized diffuse fatty metamorphosis with varying degrees of necrosis. In some areas in the liver, fatty metamorphosis was totally absent, or it involved only a few cells of the liver lobules. There were varying degrees of dilatation of the sinusoids with a considerable degree of periportal hemorrhages.

TABLE 1—Effect of Choline and Selenium on Fat Deposition in Liver

Group	Choline chloride (%)	Se *	Liver weights (Gm.)	Total liver fat† (Gm.,%)
Choline-deficient	0	0	12.1	26.9
Choline-deficient	0	1.5	3.6	17.9
Choline-deficient	0.2	0	7.2	15.9
Choline-deficient	0.2	1.5	5.5	15.5
Laboratory diet	0	0	12.0	15.2
Laboratory diet	0	1.5	11.4	16.5
Laboratory diet	0.2	0	9.6	15.2
Laboratory diet	0.2	1.5	9.9	13.2

* Intraperitoneal injections of 1.5 mg. of selenium as sodium selenate/kg. of body weight/day. † On a dry-weight basis.

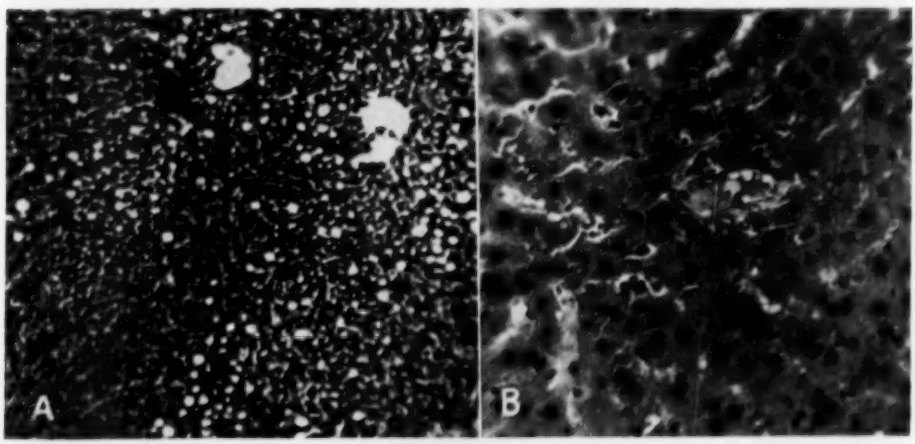


Fig. 3—Pathological changes in liver of rats fed the various diets plus selenium. Hematoxylin and eosin stain.

A) Effect of choline-deficient diet. Diffuse fatty metamorphosis in hepatic cells and nuclear hyperchromatosis. x 59.

B) Effect of choline-deficient diet plus selenium. Focal necrosis. The cell boundaries are indistinct. A few nuclei are hyperplastic, others are pyknotic. Early dilatation of sinusoids. (Rat died in 10 days.) x 295.

When the choline-deficient diet was supplemented with choline plus selenium, the additional choline did not protect the liver from selenium damage. The normal lobular structure of the liver was destroyed, the liver cells disappeared and were replaced by dilated sinusoids, and the few remaining cells were separated by edematous fluid (fig. 4A). These lesions were focal in distribution, and some areas were completely involved while others were spared.

Animals, which were on laboratory diet with or without choline plus selenium and which were destroyed at the end of the experiment, showed slightly less liver damage than did animals on the other diets. The main pathological change was dilatation of the sinusoids which were filled with nests of round cells surrounded by degenerating hepatic cells (fig. 4B). Periportal hemorrhages and irregular capillary congestion were also observed.

In the kidney, casts and tubular necrosis with calcification were the most consistent damage to animals on the choline-deficient diet with or without choline supplement plus selenium. Christensen [12] described renal changes in animals fed low choline and choline-deficient diets. Rosenfeld and Beath [7] reported tubular damage with or without calcification in chronic selenosis. However, the choline content of these diets was not determined. Therefore, whether choline deficiency played any important part in tubular damage and calcification could not be stated at the present time. The results indicate that while choline can overcome the growth-inhibiting effects of selenium to some degree, it does not protect the liver and kidney against damage by selenium.

DISCUSSION

It is doubtful that the action of choline on selenium detoxication is the result of the formation of volatile methyl compounds of selenium. Jacobi and Baumann [3] detected a characteristic garlic-like odor and observed cirrhosis in the liver of animals which received selenium in the diet, but they failed to detect any decrease in the choline content of the tissues. They stated that the amount of labile methyl used for selenium detoxication was insignificant. Our results indicate that the addition of choline to the choline-deficient and laboratory diets increased growth rate and reduced mortality but was ineffective in preventing or reducing liver and kidney damage produced by selenium. Sellers *et al.*[13] reported that supplements of methio-

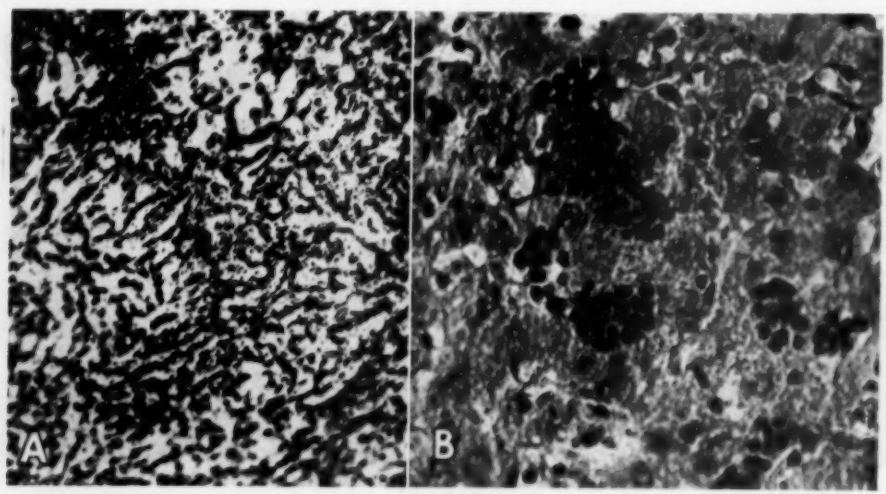


Fig. 4—Pathological changes in liver of rats fed the various diets plus selenium. Hematoxylin and eosin stain.

A) Effect of choline-deficient diet plus 0.2 per cent choline plus selenium. The lobular structure has disappeared and liver cells are replaced by dilated sinusoids. Focal necrosis. x 59.

B) Laboratory diet plus selenium. Dilated liver sinusoids filled with nests of round cells. Foci of necrotic cells. x 295.

nine and *alpha* tocopherol protected the liver against selenium damage, but that choline reduced liver damage in only 3 of the 10 selenized animals. They presented data on the average increase of body weight of animals on a basal diet plus selenium with or without choline. Their results indicated that choline added to selenized basal diet increased the body weight. In a similar experiment, methionine supplementation had no effect on the body weight. The differential effect of choline and methionine on growth rate and on liver damage would exclude the possibility of a lipotropic activity of choline at physiological levels in chronic selenosis.

In our experiments, there was a high correlation between body weight and liver weight but no correlation between liver weight and liver damage. The average weight of livers from animals on the laboratory diet with or without choline supplement and selenium showed only slight differences.

Pathological changes in the tissues studied in this experiment were not as far advanced as were those reported by Lillie and Smith [14] and Rosenfeld and Beath.[15] The extent of damage varied from animal to animal, and there was also variation within the same liver lobe. Animals which died earlier showed less liver damage than did animals which lived longer. There was no advanced cirrhosis present in any of the livers studied. It has been suggested that the toxic effects of selenium compounds probably are manifold and that more than one system is involved. From this study, it is evident that the extent of liver damage and death from selenium intoxication show no direct correlation.

It is possible that addition of more choline to the diet may reduce the tissue damage by selenium. Further studies will be carried out to determine the effects of increased choline supplementation on selenium intoxication in sheep and cattle.

SUMMARY

The effect of choline-deficient and laboratory diets with or without 0.2 per cent choline chloride on chronic selenosis was studied in young growing rats.

Animals given the choline-deficient diet plus selenium had a lower growth rate and a higher mortality rate than those on the same diet without selenium.

Selenium-injected animals showed an increased growth rate and decreased mortality rate when 0.2 per cent choline chloride was added to either the choline-deficient or laboratory diets.

The administration of selenium had no effect on the fat deposition in the liver.

Addition of 0.2 per cent choline chloride to the diets failed to protect the liver and kidney against selenium damage. The early pathological changes in the liver and kidney of the animals given selenium were discussed.

References

[1] Lewis, H. B., Schultz, J., and Gortner, R. A.: Dietary Protein and the Toxicity of Sodium Selenite in White Rats. J. Pharmacol. and Exper. Therap., *68*, (1940): 292.

[2] Rosenfeld, I., and Beath, O. A.: The Influence of Protein Diets on Selenium Poisoning. Am. J. Vet. Res., *7*, (1946): 52.

[3] Jacobi, H. P., and Baumann, C. A.: Biochemical Defect in Choline-Deficient Rats. J. Biol. Chem., *142*, (1942): 65.

[4] Fels, I. G., and Cheldelin, V. H.: Methionine in Selenium Poisoning. J. Biol. Chem., *176*, (1948): 819.

[5] Klug, H. L., Harshfield, R. D., Pengra, R. M., and Moxon, A. L.: Methionine and Selenium Toxicity. J. Nutr., *48*, (1952): 409.

[6] Halverson, A. W., Hendrick, C. M., and Olson, O. E.: Observations on the Protective Effect of Linseed Oil Meal and Some Extracts Against Chronic Selenium Poisoning in Rats. J. Nutr., *56*, (1955): 51.

[7] Rosenfeld, I., and Beath, O. A.: Pathology of Selenium Poisoning. Univ. Wyoming Exper. Sta. Bull. 275, (1946): 1–27.

[8] Smith, M. I., and Stohlmann, E. F.: Further Observations on the Influence of Dietary Protein on the Toxicity of Selenium. J. Pharmacol. and Exper. Therap., *70*, (1940): 270.

[9] Bixby, J. N., Bosch, A. J., Elvehjem, C. A., and Swanson, A. M.: Determination of Liver Fat. Agric. and Food Chem., *2*, (1954): 375.

[10] Nino-Herrera, H., Harper, A. E., and Elvehjem, C. A.: Histological Differentiation of Fatty Livers Produced by Threonine or Choline Deficiency. J. Nutr., *53*, (1954): 469.

[11] Kock-Weser, D., De la Huerga, J., and Popper, H.: Effect of Choline Supplements on Fatty Metamorphosis and Liver Cell Damage in Choline and Protein Deficiency. J. Nutr., *49*, (1953): 443.

[12] Christensen, K.: Renal Changes in the Albino Rat and Low Choline and Choline-Deficient Diets. Arch. Path., *34*, (1942): 633.

[13] Sellers, E. A., You, R. W., and Lucas, C. C.: Lipotropic Agents in Liver Damage Produced by Selenium or Carbon Tetrachloride. Proc. Soc. Exptl. Biol. and Med., *75*, (1950): 118.

[14] Lillie, R. D., and Smith, M. I.: Histogenesis of Hepatic Cirrhosis in Chronic Food Selenosis. Am. J. Path., *16*, (1940): 223.

[15] Rosenfeld, I., and Beath, O. A.: Experimentally Developed Telangiectasis and Sawdust Liver Lesions in Rats. J.A.V.M.A., *112*, (1948): 386.

A Turbidimetric Method for the Evaluation of Proteolytic Enzymes

Major SAMUEL W. THOMPSON, II, V.C., U. S. Army

Ames, Iowa

IN A CURRENT STUDY of the effects of five proteolytic enzymes on rabies virus, it was necessary for the author to devise procedures for measuring the proteolytic activity manifested by each enzyme on brain tissue. A method was needed to determine the amount of time required by a given quantity of a given proteolytic enzyme, at a given temperature, to produce maximal proteolysis of a specific substrate. The maximal limitations as to the duration and temperature of the reaction were predetermined on the basis of the known effects of these physical forces on the viability of rabies virus. The maximal quantity which could be used of any given enzyme was predetermined by known nontoxic dose levels established by intracerebral inoculation of mice.[10] Within these arbitrary limits, it was necessary to determine the minimal amount of enzyme which would produce maximal proteolysis of a brain tissue substrate.

Several methods exist for the measurement of the proteolytic activity of pure and crude enzyme preparations.[2, 5, 6] The methods of assay reported in the literature did not appear to offer a solution to the problems associated with this study.

The procedure evolved was a modification of the Klett-Summerson [4] method, for the colorimetric determination of protein in cerebrospinal fluid, which is a standard laboratory diagnostic procedure considered to be sufficiently accurate for routine purposes. The method consists of turbidimetric determinations of the percentage of decrease in the protein value of a brain tissue substrate occasioned by proteolysis.

METHODS OF PROCEDURE

Turbidimetric Method.—The modification of the Klett-Summerson method [4] for the determination of protein in cerebrospinal fluid consisted of making four colorimetric readings of 1:100 dilutions of the samples at zero hours, and at various time intervals subsequent to zero hours. The average of the readings obtained at zero hours served as the standard, and the average of each of the subsequent readings as the basis for calculation of the percentage of protein degradation.

Proteolytic Enzymes.—The five proteolytic enzymes* employed were trypsin; rhozyme P-11 and protease 15,[7] fungal and bacterial proteases, respectively; protease (trypsin),[9] which is composed of both proteases and peptidases; and proteinase A[8] which is produced from filtrates of a culture of an aerobic soil saprophyte.

Turbidimetric Determinations.—Four distinct groups of turbidimetric determinations were made as follows:

1) Determination of the Amount of Degradation Occurring in Solutions of Enzymes in the Absence of a Substrate.—Solutions of each of the enzymes—trypsin, 0.125 Gm.; rhozyme P-11, 0.1 Gm.; protease 15, 0.125 Gm.; protease (trypsin), 0.125 Gm.; and proteinase A, 2,500 units—were prepared to a total volume of 10.0 ml. in diluent A (physiological salt solution). Each solution was prepared in a sterile, stoppered test tube and thoroughly mixed by flushing ten times with a separate, sterile, 5.0-ml. pipette.

The control consisted of 10.0 ml. of diluent A mixed in a manner identical to that of the five enzyme solutions.

Colorimetric readings were made on the control and each of the five enzyme solutions immediately after preparation. These were recorded as the zero-hour readings. The control and samples were then incubated at 37 C. in a water bath. Subsequent colorimetric readings were made after four hours of incubation and again at the end of 24 hours of incubation.

2) Determination of the Amount of Degradation Occurring in Solutions of Enzymes in the Presence of a Substrate and Antibiotics.—A fresh rabbit brain was emulsified in a sterile, chilled, Waring blendor. The emulsified brain was diluted to a 20 per cent suspension, by weight, in diluent B (physiological salt solution containing 1,250 units of penicillin G and 2.5 mg. of streptomycin sulfate per milliliter). This suspension constituted the stock substrate.

Standard solutions were made to a total volume of 10.0 ml. in diluent B of each of the following enzymes: trypsin, 0.25 Gm.; rhozyme P-11, 0.2 Gm.; protease 15, 0.125 Gm.; protease (trypsin), 0.25 Gm.; and proteinase A, 5,000 units. The samples for turbidimetric determinations were pre-

Major Thompson is currently assigned to the Armed Forces Institute of Pathology, Washington, D.C.

* Trypsin was supplied by General Bio-Chemicals, Inc., rhozyme P-11 and protease 15 by Rohm and Haas Co., protease (trypsin) by Takamine Laboratory, Inc., and proteinase A by Sharp and Dohme.

pared by adding 5.0 ml. of the 20 per cent brain suspension to 5.0-ml. quantities of each of the standard solutions of enzymes. This procedure resulted in a 10 per cent suspension of brain in each of the five enzyme solutions. The final concentration of each enzyme was identical to that used in the previous group of determinations.

The controls consisted of a 10 per cent brain suspension in diluent B. This suspension was thoroughly mixed and then divided into two equal parts.

Colorimetric readings were made on the controls and each of the five samples immediately after preparation. These were recorded as the zero-hour readings. One control and the five samples were incubated at 37 C. for 24 hours. The duplicate control was held at 4 C. for a similar period of time. Colorimetric readings were made on the controls and the five samples at the end of this 24-hour period.

3) Determination of the Rate of Degradation Occurring in Solutions of Enzymes in the Presence of Antibiotics in Conjunction with the Presence or Absence of a Substrate.—A 20 per cent suspension of fresh rabbit brain in diluent B was prepared in a manner identical to that used in the second group of determinations. This suspension constituted the stock substrate.

Standard solutions of each of the five enzymes, in diluent B, were made in a manner identical to that used in the second group of determinations. Two sets of samples for turbidimetric determinations were prepared. One set consisted of 10 per cent suspensions of brain in each of the five enzyme solutions. This set was prepared in a similar manner to the samples employed in the second group of determinations. The second set of samples consisted of 5.0 ml. of each standard solution of enzyme added to 5.0 ml. of diluent B. This set of samples was similar to that used in the first group of determinations, with the exception that diluent B was employed rather than diluent A.

Two sets of controls were prepared. One set consisted of a 10 per cent suspension of brain in diluent B. This suspension was mixed in a manner identical to the test samples, and then divided into two equal parts. The other set consisted of 20.0 ml. of diluent B, which was divided into two equal parts.

Colorimetric readings were made on the two sets of controls and samples immediately after preparation. These were recorded as the zero-hour readings. A control and the five samples of each set were incubated at 37 C. for 24 hours. The duplicate controls were held at 4 C. for a similar period. Colorimetric readings were made on the controls and samples at two-, four-, six-, 12-, and 24-hour intervals, subsequent to zero hours.

4) Determination of the Amount of Degradation Occurring in Solutions of Enzymes in (*a*) the Presence and Absence of Antibiotics Without Substrate and (*b*) the Presence of Substrate with or Without Antibiotics.—Two fresh rabbit brains were separately emulsified in sterile, chilled Waring blendors. One emulsified brain was diluted to a 20 per cent suspension, by weight, in diluent A. The other brain was similarly suspended in diluent B. These two 20 per cent suspensions constituted the stock substrates.

Two sets of standard solutions of each of the five enzymes were prepared by the same method as that used in the second and third groups of determinations. One set was prepared using diluent A and the other using diluent B.

Four sets of samples for turbidimetric determinations were prepared in a manner similar to that used in the second and third groups of determinations, with the following exceptions. The first set consisted of a 10 per cent suspension of brain in diluent A in each of the five enzyme solutions in diluent A. The second set was similar to that used in the second and third groups of determinations and consisted of a 10 per cent suspension of brain in diluent B in each of the five enzyme solutions in diluent B. The third set was identical to that used in the first group of determinations and consisted of 5.0 ml. of each of the standard solutions of enzymes in diluent A. The fourth set consisted of 5.0 ml. of each of the standard solutions of enzymes in diluent B added to 5.0 ml. of diluent B.

Four sets of controls were prepared. The first and second sets consisted of a 10 per cent suspension of brain in diluents A and B, respectively. These suspensions were mixed in a manner identical to the test samples, and then divided into two equal parts per control. The third and fourth sets of controls consisted of 20.0 ml. of diluents A and B, respectively, which were each divided into two equal parts.

Colorimetric readings were made on the four sets of controls and samples immediately after preparation. These were recorded as the zero-hour readings. A control and the five samples of each of the four sets were incubated at 37 C. for four hours. The duplicate controls for the four sets of samples were held at 4 C. for the same length of time. Colorimetric readings were made at the end of the four-hour incubation period.

Calculations for Percentage of Decrease in Total Protein.—With X representing the average of the colorimetric readings at any given hour subsequent to zero hours, the percentage of decrease in the total protein value for any given sample was calculated by the formula:

$$100 - 100 \times \frac{\text{average of colorimetric readings at X hours}}{\text{average of colorimetric readings at 0 hours}} = \text{the percentage of decrease in total protein value.}$$

Calculations for Percentage of Decrease in Brain Protein.—The percentage of decrease in brain protein, for any given sample, was determined by subtracting the sum of the percentage of decrease in total protein value of the respective enzyme and substrate controls from the percentage of decrease in total protein value of the given sample.

RESULTS

The percentage of decrease in total protein calculated from the colorimetric readings made on the negative control and the five test solutions of enzymes in the absence of a substrate are shown as determination 1 (table 1). The results of this determination indicate that trypsin, rhozyme P–11, protease 15, and proteinase A evidenced no proteolytic activity when incubated at 37 C. for 24 hours in the absence of a substrate.

Protease (trypsin) did exhibit proteolytic activity when incubated at 37 C. for four hours or longer, as evidenced by a 14 per cent decrease in the protein value of the enzyme in the absence of a substrate. This indicates that protease (trypsin) probably contained some nonenzyme protein material. The manufacturer [8] stated that it was not a purified enzyme preparation.

A suspension of rabbit brain emulsion containing a standard amount of antibiotics was employed as the substrate in determination 2 (table 1). The percentages of decrease in total protein and brain protein calculated from the colorimetric readings made on the five test solutions of enzymes and negative controls are shown in the table.

By comparing the percentage of decrease in the protein value of the substrate in the presence of each enzyme with the values obtained with the negative controls, it was indicated that antibiotics in the presence of the substrate did not possess proteolytic activity.

In the series of determinations of the rate of degradation occurring in solutions of enzymes in the presence of antibiotics in conjunction with the presence or absence of a substrate, solutions of enzymes and negative controls similar to those employed in determination 2 were used as one set of test solutions. These were compared with solutions of enzymes and negative controls containing identical quantities of antibiotics in the absence of a brain emulsion substrate. The percentage of decrease in total protein and brain protein, when applicable, was calculated from the colorimetric readings made at two-, four-, six-, 12-, and 24-hour intervals on each test solution. The protein values for the fourth

TABLE 1—Decrease in Protein Values Occurring in Five Enzyme Solutions Incubated at 37 C. in the Presence and Absence of Antibiotics and the Presence of Brain Tissue with and Without Antibiotics

Test solutions	Percentage of decrease in protein values											
	Total protein								Brain protein			
Substrate:	No substrate				Brain tissue				Brain tissue			
Antibiotics:	Present		Absent		Present		Absent		Present		Absent	
Hours at 37 C.:	4	24	4	24	4	24	4	24	4	24	4	24
Determination 1												
Trypsin	—	—	0	0	....	....	....	....	....	....	....	....
Rhozyme P–11	—	—	0	0	....	....	....	....	....	....	....	....
Protease 15	—	—	0	0	....	....	....	....	....	....	....	....
Protease (trypsin)	—	—	14	14	....	....	....	....	....	....	....	....
Proteinase A	—	—	0	0	....	....	....	....	....	....	....	....
Determination 2												
Trypsin	....	....	....	....	—	17	—	—	—	17	—	—
Rhozyme P-11	....	....	....	....	—	20	—	—	—	20	—	—
Protease 15	....	....	....	....	—	14	—	—	—	14	—	—
Protease (trypsin)	....	....	....	....	—	26	—	—	—	12	—	—
Proteinase A	....	....	....	....	—	7	—	—	—	7	—	—
Determination 3												
Trypsin	0	0	—	—	15	15	—	—	15	15	—	—
Rhozyme P-11	0	0	—	—	19	19	—	—	19	19	—	—
Protease 15	0	0	—	—	11	11	—	—	11	11	—	—
Protease (trypsin)	14	14	—	—	26	26	—	—	12	12	—	—
Proteinase A	0	0	—	—	9	9	—	—	9	9	—	—
Determination 4												
Trypsin	0	—	0	—	18	—	18	—	18	—	18	—
Rhozyme P–11	0	—	0	—	20	—	20	—	20	—	20	—
Protease 15	0	—	0	—	11	—	11	—	11	—	11	—
Protease (trypsin)	12	—	12	—	26	—	25	—	14	—	13	—
Proteinase A	0	—	0	—	9	—	9	—	9	—	9	—
Determinations 1, 2, 3, and 4												
37 C. neg. control	0	0	0	0	0	0	0	0	0	0	0	0
4 C. neg. control	0	0	0	0	0	0	0	0	0	0	0	0

and twenty-fourth hour are shown as determination 3 (table 1).

None of the five enzymes tested produced any measurable amount of proteolysis by the end of the second hour. Maximum proteolysis occurred between the second and fourth hour of incubation. No increase in the amount of proteolysis was detectable after the fourth hour of incubation.

A comparison of the results obtained with the five enzymes in the presence of brain tissue and antibiotics with the results obtained with antibiotics without brain tissue indicate that the antibiotics did not exert any detectable influence on the proteolytic activity of the enzymes. These results tend to confirm those of determination 2.

The fourth group of determinations (table 1) constituted a recheck of the previous three groups of determinations. Four series of suspensions of identical quantities of enzymes used in the previous three groups of determinations were employed as test solutions. The decrease in total protein and brain protein, when applicable, occurring in each series of suspensions and controls after four hours incubation at 37 C. was determined.

The results obtained with the five enzymes in the absence of a substrate and antibiotics in determination 4 confirm the results shown for determination 1. These collective results indicate more definitely that, with the exception of protease (trypsin), none of the enzymes evidenced proteolytic activity when incubated at 37 C. in the absence of a substrate. In determination 4, it was again indicated that the proteolytic activity of the five enzymes is not detectably influenced by the presence of antibiotics, either alone or in the presence of brain tissue.

In determination 4, the results obtained with the five enzymes in the presence of brain tissue were similar to those obtained in determinations 2 and 3. In each of these groups of determinations, it was apparent that with all five enzymes, maximum proteolysis occurred by the fourth hour of incubation at 37 C. No increase in the amount of proteolysis was detectable after the fourth hour of incubation.

The results shown in table 1 also tend to indicate the degree of proteolytic effectiveness of each enzyme preparation. Trypsin and rhozyme P–11 appear to be more effective than protease 15, protease (trypsin), and proteinase A. Protease 15 and protease (trypsin) were equally efficient. Proteinase A was the least effective of the five enzymes tested. The procedures employed in each turbidimetric determination of the proteolytic activity of the five enzymes were repeated a sufficient number of times to ascertain that the results obtained were consistent.

Discussion

In the series of turbidimetric determinations conducted in this study, the pH of each proteolytic enzyme reaction was ascertained to be in the range of 7.4 to 7.6. Although this pH range was not the optimal pH for each enzyme used, it was within the effective range of each of the five enzymes.[7–9] According to Bodansky,[1] the activity of an enzyme depends upon the pH at which the reaction takes place. At a certain pH, the enzyme may undergo destruction while it acts on the substrate. On the other hand, even though there is no destruction of the enzyme, the rate at which it acts depends on the pH of the solution. There is a certain pH for every enzyme at which the reaction velocity is optimal. It is apparent that the pH, had it been at an optimal concentration for each enzyme, could have resulted in either a greater degree of proteolysis or a decrease in the reaction time.

The incubation temperature of each proteolytic enzyme reaction conducted for the turbidimetric determinations was held at 37 C., since temperature also has a definite relationship to the velocity of an enzyme reaction. For each enzyme, there is an optimal temperature range at which the reaction velocity is greatest. Grob,[3] in his discussion of conditions which affect the activity of proteolytic enzymes, states that most of these enzymes are very active at 37 C., slightly increased in activity by short exposure to a temperature between 37 and 60 C., slightly impaired by a temperature of 65 C., and destroyed, if in solution, above 70 C. The necessary temperature varies inversely with the length of time of the reaction. The temperature of 37 C. was within the effective temperature range of each of the five proteolytic enzymes tested. This temperature was selected because the enzymes under investigation were intended for later use in the

study of their effects on rabies virus. It was essential to assay the activity of the enzymes in relation to their subsequent utilization. From a review of the literature as to the effect of heat on rabies virus, it appeared that 37 C. was the highest temperature which could be employed without adversely affecting the virus while, at the same time, providing an effective temperature for the enzyme reactions.

The results obtained in the turbidimetric determinations correlated well with the factors, as described by Bodansky [1] and Grob,[3] which control the velocity of enzyme reactions, such as: concentration of substrate, concentration of enzyme, pH of the reaction, and inactivation of the enzyme. The belief that the decrease in the protein value of the substrate was a direct indication of the proteolytic activity of the enzymes tested is based on the following factors: significant decreases in the protein value of the substrate were recorded when the substrate was incubated at 37 C. for four to 24 hours in the presence of each enzyme; no decrease in the protein value of the substrate was recorded when the substrate was handled in a similar manner in the absence of enzyme.

By means of this turbidimetric procedure, it was possible to determine that each of the five proteolytic enzymes used in this study were capable of degrading brain protein when employed in concentrations that would not produce proteolytic enzyme shock [10] in mice. The procedure also made it possible to ascertain the amount of time required at 37 C. for each enzyme to accomplish maximum proteolysis of the brain tissue. In this regard, it was particularly interesting to find that with each enzyme-brain emulsion preparation, the end point of the reaction was reached by the fourth hour of incubation at 37 C. This finding made it possible to establish a standard incubation schedule of four hours at 37 C. for the subsequent investigations of the effect of these enzymes on rabies virus.

CONCLUSIONS

In view of the results obtained with the modified Klett-Summerson method for the colorimetric determination of protein, it is felt that this method might be suitable for assaying the activity of proteolytic enzymes on any suitable substrate. The method could lend itself to the determination of the influence on the rate of proteolysis of various factors which might influence the velocity of a given proteolytic enzyme reaction.

SUMMARY

A turbidimetric method for the assay of the velocity of proteolytic enzyme reactions, under given conditions which effect this rate of velocity, was described. By means of this method, trypsin, rhozyme P–11, protease 15, protease (trypsin), and proteinase A were assayed for their proteolytic effect on a brain tissue substrate. Significant decreases in the protein content of the substrate were produced by proteolysis with each of the five enzymes. In the case of all five enzymes, no detectable proteolysis occurred until after the second hour at 37 C. No additional increase in the amount of proteolysis occurred after the fourth hour.

References

[1] Bodansky, M.: Introduction to Physiological Chemistry. 4th ed. John Wiley and Sons, Inc., New York, 1938.

[2] Christensen, L. R.: Methods for Measuring the Activity of Components of the Streptococcal Fibrinolytic System, and Streptococcal Desoxyribonuclease. J. Clin. Invest., *28*, (1949): 163–172.

[3] Grob, D.: Proteolytic Enzymes, the Control of Their Activity. J. Gen. Physiol., *29*, (1946): 219–247.

[4] Klett Manufacturing Co.: The Determination of Protein in Cerebrospinal Fluid, in "The Clinical Manual and General Directions for the Klett-Summerson Photoelectric Colorimeter. Klett Manufacturing Co., ca. 1952.

[5] Kunitz, M.: Crystalline Soybean Trypsin Inhibitor. J. Gen. Physiol., *30*, (1946): 291–310.

[6] Northrop, J. H.: Action of Papain and Ficin on Tadpoles and Arbacia Eggs. J. Gen. Physiol., *30*, (1947): 375–376.

[7] Reed, G.: Personal communication, 1955.

[8] Scheidy, S. F.: Personal communication, 1955.

[9] Takamine Laboratory, Inc.: Protease (Trypsin), Takamine Laboratory, Inc., Clifton, N.Y., 1954.

[10] Thompson, S. W.: Toxicity of Various Enzymes when Inoculated Intracerebrally into Mice. Cornell Vet. (in press).

The Intradermal Sarcocystin Test

F. I. AWAD, Ph.D., B.V.Sc., M.R.C.V.S.

Khartoum, Sudan

In view of the promising results obtained from the application of the skin tests in the diagnosis of some bacterial, viral, mycotic, and parasitic diseases, it was decided to investigate the possibility of applying the skin test in the diagnosis of sarcosporidiosis.

Materials and Methods

Preparation of the Antigen.—Sheep esophagi infected with macroscopic sarcocysts were collected from the abattoir. The cysts were excised, collected in a sterile container, weighed, and a 10.0 per cent suspension of their contents was prepared in saline. This was quickly frozen in a CO_2 refrigerator and rapidly thawed; this process was repeated three times. After 15 hours in an ordinary refrigerator, the suspension was centrifuged at 4,300 r.p.m. for two hours. The clear supernatant fluid was removed and normal saline containing 0.1 per cent merthiolate was added to give an ultimate concentration of merthiolate of 1:10,000. This constituted the final antigen for the skin test. It was frozen and stored at low temperatures.

Technique.—(1) After epilation, an area of skin in the region of the flank or back was washed with warm water and dried. (2) Before injection, the thickness of the skin at the site of inoculation was measured with a caliper. (3) The test dose of 0.1 ml. of sarcocystin was inoculated intradermally and the same quantity of the diluent was injected about 1 inch away for comparison. The reaction was observed 24, 48, and 72 hours later.

Experimental Results

A) Assay of Antigen on Artificially Immunized Guinea Pigs.—The test was carried out on 6 adult male guinea pigs which had been artificially immunized by injecting each intraperitoneally with 0.1 ml. of sarcocyst antigen, which was repeated a week later. The skin test was carried out a week after the second inoculation. Six other guinea pigs were used as controls.

TABLE 1—Results of Intradermal Sarcocystin Test on Artificially Immunized Guinea Pigs

Animal No.	Reaction to sarcocystin		Reaction to saline	
	Erythema (cm.)	Edema (cm.)	Erythema (cm.)	Edema (cm.)
1	1.7	3.1	None	None
2	2.0	2.8	0.2	0.4
3	1.2	1.7	None	None
4	1.0	2.2	None	0.2
5	1.3	3.0	0.3	0.4
6	1.9	2.6	None	None

In 24 hours after inoculation, the immunized animals showed an inflammatory edema and erythema at the site of injection. The reaction appeared to reach its maximum 48 hours after inoculation when the hemorrhagic area appeared brownish red surrounded by hyperemia; edema and induration were still marked. On the third day, the reaction began to subside and disappeared within the week. In the control guinea pigs, there was no noticeable reaction. The measurements obtained of the erythema and edema at the end of a 48-hour period are shown (table 1).

A test was regarded positive when erythema and edema measured 1.0 to 2.0 cm. in diameter and doubtful when there was slight erythema and a trace of edema measuring 0.5 cm. or less in diameter at the end of the 48 hours.

B) Determination of the Optimal Dilution of the Antigen.—Six artificially immunized guinea pigs were placed in three groups of 2 each. Each animal received 0.1 ml. of sarcocystin dilution as follows: group A, 1:100; group B, 1:200; and group C, 1:400.

The 1:200 antigen dilution gave the maximal reaction at the end of 48 hours.

C) The Intradermal Sarcocystin Test on Guinea Pigs Fed on Sarcocystis Tenella.—The test was carried out on 12 guinea pigs. Serums were collected for the complement-fixation test (CF) (Awad and Lainson [2]) and the dye test (Awad [1]) 143 days after the last feeding of *S. tenella*. Two days later the skin test was carried out.

From the Veterinary Research Division, Department of Animal Production, Ministry of Animal Resources, Khartoum, Sudan.

The author expresses his gratitude to Professor P. C. C. Garnham, London School of Hygiene and Tropical Medicine, under whose supervision this work was carried out, and to Dr. A. B. Erickson, University of Minnesota, for supplying the infected cottontail rabbit.

TABLE 2—Results of Complement-Fixation (CF), Dye, and Skin Tests on Guinea Pigs Fed Sarcocystis Tenella

Animal No.	CF test	Dye test	Skin test
1	—	+	+
2	+	+	+
3	—	+	—
4	—	—	—
5	+	+	—
6	+	+	+
7	—	+	+
8	+	+	+
9	—	+	+
10	—	+	+
11	—	—	—
12	—	+	+

The results of the three tests are shown (table 2).

Of the 12 animals, 8 reacted positively to the skin test, 6 of which were positive in the absence of detectable infection; 10 animals reacted positively to the dye test, 7 of which were positive in the absence of detectable infection; and 4 animals reacted positively to the CF test, 1 of which was positive in the absence of detectable infection.

These results indicated a correlation between the positive reactors to the skin test and the dye test; such a correlation did not exist between the skin test and the CF test. No animal gave a positive skin test in the absence of the cytoplasm-modifying antibody.

TABLE 3—Results of Complement-Fixation, Dye, and Skin Tests on Rabbits Fed Sarcocystis Leporum

Animal No.	CF test	Dye test	Skin test
1	—	—	—
2	—	+	+
3	—	+	+
4	+	+	+

D) The Intradermal Sarcocystin Test on Rabbits Fed on Sarcocystis Leporum.—The tests were carried out on 4 rabbits, as outlined in experiment C. The results are shown (table 3).

The infection was only demonstrated in rabbit 4, which gave positive results to the three tests. Here again, there is a correlation between the skin test and the dye test, but not with the CF test.

DISCUSSION

In the present investigation, the intradermal sarcocystin test was carried out on experimentally infected animals.

Occasionally, an animal reacted in a nonspecific manner, reacting equally to sarcocystin and the control diluent. The reaction began to subside on the third day and disappeared by the seventh day. The reaction was of the delayed type, similar to those observed in mild tuberculin reactions.

The skin test does not necessarily indicate infection, since mere contact or inoculating the infective agent apparently may sensitize the animal to the test. Taliaferro[4] also found that animals immunized with parasitic extracts may react.

Fisher[3] suggested that the presence of sensitivity to toxoplasmin together with a positive dye test signifies a previous infection with Toxoplasma, but that a significant CF test titer is probably associated with extensive, often clinical, evidence of toxoplasmosis. This may be equally true of sarcosporidiosis, as evidenced by the experimental findings.

Analysis of the results showed that the incidence of positive skin reactions is so great that the diagnostic value of the test is not great. However, it can be used as a screening test. Only if sensitivity is present should other serological methods be used.

As sarcocystin is highly toxic to experimental animals (Awad[5]), the test should not be attempted on human beings in its present form.

SUMMARY

1) The present study describes the possibility of using dermal sensitivity in a diagnostic test for sarcosporidiosis.

2) The reaction obtained was of the delayed type; an animal was considered positive when erythema and edema measured 1 to 2 cm. in diameter at the end of 48 hours.

3) It is concluded that positive reactions to the skin test often occur in the absence of established infection. While it has little specific diagnostic value, it can be used as a screening test.

References

[1] Awad, F. I.: A New Dye Test for Toxoplasma and Sarcocystis Infections. Tr. Roy. Soc. Trop. Med. and Hyg., *48*, (1954): 337–341.

[2] Awad, F. I., and Lainson, R.: A Note on the Serology of Sarcosporidiosis and Toxoplasmosis. J. Clin Path., *7*, (1954): 152–156.

[3] Fisher, O. D.: A Survey with Toxoplasmin Intradermal Antigen. Lancet, *2*, (1951): 904.

[4] Taliaferro, W.: The Immunology of Parasitic Infection. London (1939): 263.

[5] Awad, F. I.: Unpublished data.

An Improved Method for the Rapid Cultivation of Large Yields of *Vibrio Fetus*

GORDON W. ROBERTSTAD, M.S., and S. M. MORRISON, Ph.D.

Fort Collins, Colorado

NUMEROUS TECHNIQUES for the propagation of *Vibrio fetus* to be used for animal inoculations and antigen preparations have been reported. These procedures have all required a prolonged incubation period and large quantities of materials in order to produce a satisfactory yield of cells. This paper presents a *V. fetus* culture method which produces a large yield of cells in 24 to 28 hours.

McFadyean and Stockman,[4] in their studies on vibrionic abortion, experienced difficulty in obtaining adequate growth of *V. fetus*. Several investigators[5,7] have utilized solid thiol agar (Difco) which requires 96 hours to give an adequate cell yield when incubated at 37 C. in an atmosphere of 10 per cent CO_2. Firehammer and Marsh[2] observed that *V. fetus* retained its normal morphology and its motility for a longer period in a fluid medium than on a solid medium. However, these authors recommended the utilization of a modified Huntoon's "hormone" agar to obtain sufficient cells in a 48-hour incubation period.

Hansen *et al.*[3] used solid thioglycollate medium to prepare antigens for the Vibrio-agglutination test. The cells were harvested after a 72- to 96-hour incubation period. They also reported that 300-ml. Erlenmeyer flasks which contained 100 ml. of thioglycollate broth could be used to produce large quantities of organisms in two to three days if a heavy inoculum was used. Wiidik and Hlidar[8] incubated cattle isolates of *V. fetus* for 96 hours when cultivated on chocolate agar medium. Ristic *et al.*[6] also incubated their cultures for 96 hours when the organism was propagated on Brucella agar (Albimi).

MATERIALS AND METHODS

Seed cultures were prepared by inoculating tubes of semisolid Brucella broth (1.0 Gm. of agar and 0.05 Gm. of glutathione added per liter) with organisms from the stock culture. The tubes were incubated at 37 C. for 48 hours in a desiccator in which 10 per cent of the air had been replaced with nitrogen. The surface growth was transferred to 100 ml. of Brucella broth (0.05 Gm. of glutathione added per liter) contained in a 250-ml., cotton-stoppered, Erlenmeyer flask. The flask was placed in a desiccator and 60 per cent of the air was removed by vacuum pump and replaced with nitrogen. The desiccator was opened and the flask mouth was sealed with a piece of aluminum foil held firmly in place with a rubber band. The flask was incubated at 37 C. for 24 hours on a gyratory shaker (New Brunswick model 83) at 140 gyrations per minute. The culture purity was determined microscopically and the growth was used to inoculate additional flasks—2.5 ml. to each flask of broth. These flasks were again incubated on the shaker in the manner described. Cells were harvested by centrifugation after 24 hours' incubation.

For the determination of the time-growth curve utilizing the shaker method, a fresh isolate, 021, from an aborted lamb fetus was used. Isolated colonies on blood agar plates were transferred to tubes of semisolid Brucella broth. After 48 hours' incubation, the resulting growth was introduced into Erlenmeyer flasks and incubated as previously outlined. After 24 hours, the contents of two of these flasks were combined to serve as inoculum for the time-growth curve determination. The cell suspension had a light transmittance of 25 per cent as determined with a B and L spectronic 20 colorimeter (650-mμ wave length, red filter). An inoculum of 2.5 ml. of this suspension was used for each of 42 flasks of Brucella broth. Sixty per cent of the air was replaced with nitrogen after which the mouth of each flask was sealed with aluminum foil. Three inoculated flasks were used for zero time determinations and 21 of the flasks were incubated at 37 C. on the gyratory shaker for varying lengths of time.

The percentage of light transmittance, pH determination, and microscopic examination of gram-stained preparations were made of three flasks at each of the following time intervals: four, eight, 12, 24, 28, 32, and 48 hours.

The remaining 18 flasks served as controls and were incubated in a stationary position at 37 C. Data were obtained on this group at four, eight, 12, 24, 40, and 48 hours.

From the School of Veterinary Medicine and the Experiment Station of Colorado A. & M. College, Fort Collins.

This study was an integral part of a western regional research project, W-27, on vibriosis of sheep.

The authors acknowledge the aid furnished by the Colorado A. & M. Research Foundation.

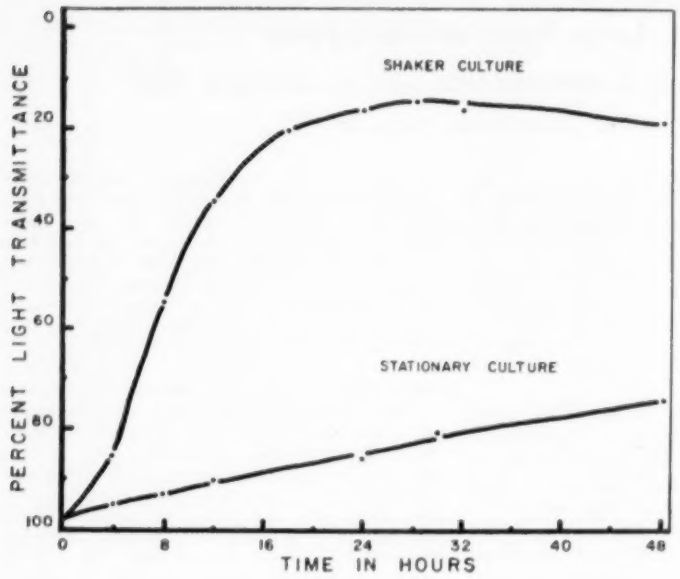


Fig. 1—Time-growth curves of Vibrio fetus (isolate 021).

RESULTS AND DISCUSSION

The comparison of light-transmittance readings of Brucella broth cultures of *V. fetus*, 021, incubated for varying lengths of time with and without agitation is shown (fig. 1). It is apparent that gyratory shaking at 140 gyrations per minute enhances the growth of this organism to rapidly produce high yields of cells. Whereas the cultures grown with the shaker technique reached minimum light transmittance (13%) in 28 hours, the stationary control flasks only attained a light transmittance of 73 per cent at the final reading (48 hours).

The *V. fetus* culture used was a freshly isolated, catalase-positive, H_2S-negative, gram-negative, short, curved rod. With the shaking technique, the original morphology remained constant through the 32-hour incubation period. Evidence of autolysis was apparent at the 48-hour microscopic examination. The pH remained constant at 7.1 through the 12-hour readings but became progressively more alkaline until a pH of 7.7 was reached at 48 hours.

Three other recent isolates (1 ovine, 2 bovine) responded similarly to this method of growth. Some of the isolates which had been propagated in the stock culture collection over a period of several years did not grow in the shaker flasks on initial trial. With the exception of a few "mucoid"-type isolates, these old cultures grew on the shaker after an initial stationary incubation period of 24 hours. Once these cultures were adapted to growth on the shaker they maintained the ability to grow rapidly.

The growth of *V. fetus* in Brucella broth when incubated on the shaker was of uniform turbidity throughout the medium. There was no settling of cells, even after standing for 24 hours or more. Those cultures which could not be induced to grow on the shaker gave a mucoid-type reaction when examined by the acriflavine-agglutination test,[1] while the isolates that grew well demonstrated the smooth-type reaction.

It should be emphasized that cultures used for inoculating the shaker flasks should be no older than 72 hours. Older cultures gave inconsistent results.

The growth response of *V. fetus* to agitation may be a function of several factors: (*a*) better contact of the cells with the nutrients, (*b*) more efficient removal of toxic products from the vicinity of the bacteria, and (*c*) a more constant physical environment for each of the cells.

SUMMARY

A shaker technique has been successfully applied to the propagation of *Vibrio fetus* for the production of a high yield of cells in a short period.

References

[1] Braun, W., and Bonestell, A.: Independent Variation of Characteristics in *Brucella Abortus* Variants and Their Detection. Am. J. Vet. Res., *8*, (1947): 386–390.

[2] Firehammer, B. D., and Marsh, H.: Preparation of Antigen for Detection of Agglutinins for *Vibrio Fetus*. Am. J. Vet. Res., *14*, (1953): 392–395.

[3] Hansen, P. A., Price, K. E., and Clements, M. F.: Suitable Thioglycollate Media for the Cultivation of *Vibrio Fetus*. J. Bact., *64*, (1952): 772.

[4] McFadyean, J., and Stockman, S.: Report of the Departmental Committee Appointed by the Board of Agriculture and Fisheries to Inquire into Epizootic Abortion. Appendix to Part III, Abortion in Sheep, London, 1913.

[5] Plastridge, W. N., and Williams, L. F.: Procedure for Conducting the Tube Agglutination Test for Vibriosis. Third Revision. Storrs Agric. Exper. Sta. Bull. 20, 1950.

[6] Ristic, M., Sanders, D. A., and Tyler, M. E.: *Vibrio Fetus* Infection in Cattle. I. A Comparative Study of the Agglutinin Response in Cervicovaginal Mucus and Blood Serum to Homologous Antigens Following Abortion. Am. J. Vet. Res., *16*, (1955): 246–250.

[7] Stockton, J. J., and Newman, J. P.: The Preservation of *Vibrio Fetus* by Lyophilization. Cornell Vet., *40*, (1950): 377–379.

[8] Wiidik, R. W., and Hlidar, G. E.: Untersuchungen über die antigene Struktur von *Vibrio fetus* vom Rind. Das Kapsel, oder K-antigen von *Vibrio fetus*. Zentralbl. f. Vet.-med., *2*, (1955): 238–250.

Antigenic Differences Among Strains of *Leptospira Pomona*

AARON D. ALEXANDER, M.S.; LARUE B. EVANS, B.S.; RUTH MARCHWICKI, B.S.

Washington, D.C.

LEPTOSPIRA POMONA was first isolated from a dairy farmer in Australia by Clayton and Derrick.[1] Since that time, its worldwide distribution among larger domestic animals and its infection potential for man has been repeatedly demonstrated.[2] *Leptospira pomona* has been less frequently isolated from dogs,[3,4] rodents,[5–8] and various other wildlife species.[9,10]

All strains of *L. pomona* are believed to be antigenically homologous and, consequently, only one serotype has been recognized in Wolff and Broom's proposed classification of pathogenic leptospiras.[11] The antigenic similarity of leptospiral isolates from two different epizootics of bovine leptospirosis in the United States and *L. pomona* was first demonstrated by Gochenour *et al.*[12] In a recent monograph, Wiesmann[13] included results of agglutinin-absorption studies which reportedly demonstrate antigenic differences in two *L. pomona* strains isolated in this country. However, this conclusion is equivocal on the basis of the presented data. Cross absorptions by the two antigenically dissimilar strains reduce homologous antiserum titers to the same level as absorptions by the respective homologous antigens.

Variations in the cross-reactive patterns of various American strains of *L. pomona* against a battery of serotype-specific antiserums were frequently observed in this laboratory and afforded ancillary evidence of antigenic dissimilarities. Minor differences in antigenic composition of *L. pomona* strains were observed in the early study by Gochenour *et al.*[12] in this laboratory. Recently, during the course of serological studies on Malayan leptospiras, antigenic differences in *L. pomona* strains were again seen.[14] In view of this, a study of the antigenic characteristics of *L. pomona* strains was initiated. The finding of antigenic differences among *L. pomona* strains is reported in this paper.

MATERIALS AND METHODS

Twenty-three strains of *L. pomona*, including the type strain originally isolated in Australia,[1] were studied. The source, designation, and place of isolation of the strains studied are shown (table 1). The method of preparing antiserums in rabbits, the microscopic agglutination (agglutination-lysis) test procedure, and agglutinin-absorption technique employing formalin-treated packed leptospiras are described elsewhere in detail.[15]

RESULTS

The initial testing for antigenic differences among the 23 *L. pomona* strains was based upon the cross agglutination-lysis reactions of these strains employed as antigens against a collection of antigenically distinct type, leptospiral rabbit antiserums. For this screening, antiserums against 17 different serotypes which are routinely used for culture typing were employed.[24] In addition, antiserums against seven different strains of *L. pomona* and three serotype members of the *autumnalis* group were also selected to screen the strains under study. The *autumnalis* serotypes were selected because they cross agglutinate with *L. pomona*.

Except for minor differences in cross reactions against *Leptospira sentot; Leptospira djasiman; Leptospira australis A; Leptospira autumnalis*, Akiyami A; and *Leptospira autumnalis*, Fort Bragg, the agglutination reactions of the *L. pomona* strains were essentially identical. Based on these minor differences in cross agglutinations, the strains could be grouped under five different patterns of reactions. The cross-agglutination reactions of the strains are shown (table 2).

In addition to the five representative strains shown in table 2, strains Y–H and LC–34 were selected for cross agglutinin-absorption studies. Strain Y–H was employed because it was the sole isolate from a horse and strain LC–34 was included because it was isolated from a cow that demonstrated high titer agglutinins against *Leptospira sejroe*. The results of agglutinin-absorption studies are shown (table 3). The agglutinin-absorption test results on strains Y–H and LC–34 essentially paralleled the findings with strains Pomona and

From the Division of Veterinary Medicine, Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D.C.

TABLE 1—Source of Leptospira Pomona Strains

Strain	Isolation: Host	Isolation: Place	Isolation: Year	Source or reference
Pomona	Human	Australia	1937	Clayton and Derrick [1]
C–B	Bovine	New York	1952	T. Burnstein, New York State Vet. Coll., Ithaca, N.Y.
V–42	Bovine	West Virginia	1952	Vet. Div., Walter Reed Army Inst. Res., Washington, D.C.
S–91	Porcine	Indiana	1951	Gochenour *et al.*[16]
Y–H	Equine	New York	1952	Roberts *et al.*[17]
LC–34	Bovine	Florida	1953	Galton *et al.*[18]
C–164	Bovine	New Jersey	1946	Baker and Little [19]
T–262	Bovine	New Jersey	1946	Baker and Little [19]
K M	Bovine	California	1951	G. W. Hooper Found., San Francisco, Calif.
Tatnall	Porcine	Georgia	1952	Coastal Plain Exper. Sta., Tifton, Ga.
Sippel	Bovine	Georgia	1951	Sippel *et al.*[20]
NYA	Bovine	New York	1949	Reinhard *et al.*[21]
NYB	Bovine	New York	1949	Reinhard *et al.*[21]
344	Bovine	Virginia	1952	Virginia Polytech. Inst., Blacksburg, Va.
643	Porcine	Unknown	Unknown	Pitman-Moore Co., Zionsville, Ind.
215	Porcine	Unknown	Unknown	Pitman-Moore Co., Zionsville, Ind.
357–R	Bovine	New York	1953	K. M. Lee, New York State Vet. Coll., Ithaca, N.Y.
Ill. hog	Porcine	Illinois	1953	Bryan *et al.*[22]
LC–73	Bovine	Florida	1954	Galton *et al.*[18]
LC–80	Bovine	Florida	1954	Galton *et al.*[18]
LC–78	Bovine	Florida	1954	Galton *et al.*[18]
Lamb	Human	Malaya	1954	Alexander *et al.*[14]
Suis	Porcine	Argentina	1944	Savino and Renella [23]

V–42, respectively, and therefore were not included in this table.

Within the limitations of the methods employed, it is apparent that antigenically dissimilar strains comprise the serogroup *pomona*. Strains C–164 and S–91 contain antigenic components that are specific for these respective strains and that are not shared by the other strains. The antigenic inter-relationships of strains V–42, Pomona, and C–B are particularly noteworthy. From the results obtained, strain Pomona apparently intergrades in antigenic complexity between strains V–42 and C–B. Thus, compared to the type strain Pomona, strain V–42 is an "incomplete biotype"—that is, it contains part but not all of the antigenic components of strain Pomona. Similarly, strain Pomona as well as strain V–42 are incomplete biotypes of strain C–B. However, cross agglutinins in antiserum C–B against strain Pomona were completely absorbed by antigen V–42. This was inconsistent with the otherwise observed intergrading relationships of these three strains. Consequently, additional cross agglutinin-absorption studies were conducted on strains V–42, Pomona, and C–B. The results of the additional studies were essentially identical to the initial ob-

TABLE 2—Cross-Agglutination Reaction Patterns of Leptospira Pomona Strains

Strain	Rabbit antiserum*: Homologous titer	Reciprocal of titer against *L. pomona* strain: Pomona†	C–B	C–164‡	V–42§	S–91
L. djasiman	25,600	—	100	—	—	—
L. sentot	25,600	—	100	100	100	100
L. australis A	6,400	—	100	—	—	100
L. autumnalis, Fort Bragg	6,400	—	400	400	400	400
L. autumnalis, Akiyama A	6,400	—	100	—	400	100
L. bangkinang	6,400	—	—	—	—	—
L. rachmat	6,400	400	1,600	1,600	1,600	1,600
L. pomona, Pomona	6,400	6,400	6,400	25,600	25,600	6,400
L. pomona, V–42	25,600	6,400	25,600	25,600	25,600	25,600
L. pomona, LC–34	6,400	1,600	6,400	6,400	6,400	6,400
L. pomona, S–91	6,400	6,400	6,400	6,400	6,400	6,400
L. pomona, C–164	25,600	6,400	6,400	25,600	25,600	6,400
L. pomona, NYA	6,400	1,600	6,400	6,400	6,400	6,400
L. pomona, KM	6,400	6,400	6,400	25,600	6,400	6,400
L. pomona, Lamb	25,600	6,400	6,400	25,600	25,600	6,400

* All strains negative (— = <1:100) against *L. andaman*, *L. semaranga*, *L. djatzi*, *L. hyos*, *L. ballum*, *L. canicola*, *L. icterohaemorrhagiae*, *L. australis B*, *L. alexi*, *L. grippotyphosa*, *L. medanensis*, and *L. javanica*.

† Strains suis and Sippel showed same cross reactions.

‡ Strain Y–H showed same cross reactions.

§ Strains LC–34, T–262, KM, Tatnall, NYA, NYB, 344, 643, 215, 357–R, Ill. hog, LC–73, LC–80, LC–78, and Lamb, showed same cross reactions.

TABLE 3—Cross Agglutinin-Absorption Tests on Selected Strains of Leptospira Pomona

Rabbit antiserum		Reciprocal of titer against *L. pomona* test antigen				
Against	Absorbed with	C–B	Pomona	V–42	8–91	C–164
L. pomona, C–B	Unabsorbed	25,600	6,400	25,600	6,400	25,600
	C–B	—‡	—	—	—	—
	Pomona	6,400	—	—	—	—
	V–42	6,400	—	—	—	—
	8–91	1,600	100	—	—	—
	C–164	1,600	—	—	—	—
L. pomona, Pomona*	Unabsorbed	6,400	6,400	6,400	6,400	6,400
	C–B	—	—	—	—	—
	Pomona	—	—	—	—	—
	V–42	400§	400§	—	400	100
	8–91	1,600	400	100	—	400
	C–164	1,600	400	—	—	—
L. pomona, V–42	Unabsorbed	102,400	25,600	25,600	25,600	102,400
	C–B	—	—	—	—	—
	Pomona	—	—	—	—	—
	V–42	—	—	—	—	—
	8–91	1,600	400	100	—	400
	C–164	400	—	400	—	—
L. pomona, 8–91	Unabsorbed	25,600	6,400	25,600	25,600	25,600
	C–B	—	—	—	100	—
	Pomona	—	—	—	100	—
	V–42	400	—	—	400	—
	8–91	—	—	—	—	—
	C–164	100	—	—	400	—
L. pomona, C–164	Unabsorbed	25,600	6,400	25,600	25,600	25,600
	C–B†	400	—	—	—	400
	Pomona	—	—	—	—	400
	V–42	1,600	400	—	—	400
	8–91	1,600	1,600	400	—	1,600
	C–164	100	—	—	—	—

* Type strain.
† Incompletely absorbed.
‡ — = <1:100.
§ Residual agglutinins were removed after repeated absorptions.

servations; however, strains Pomona and V–42 were indistinguishable after repeated absorption treatments of aliquots of each of the three antiserums with the three different antigens. The apparent antigenic differences between strains V–42 and Pomona may be quantitative rather than qualitative.

DISCUSSION

The cross-agglutination screening of leptospiral strains against a battery of serotype rabbit antiserums that encompass the extensive antigenic spectrum of leptospiras, as well as against antigenically related serotypes, provides important clues for subsequent definitive identification through agglutinin-absorption procedures. In this study, although there were marked similarities in the over-all cross reactions of the strains, four antigenically dissimilar strains of *L. pomona* were revealed on the basis of minor differences in cross-agglutination reactions.

It should be noted, however, that uniform cross-reactive patterns by serologically heterologous members of the same serogroup and, conversely, minor differences in cross reactions of homologous strains have been observed in previous classification studies conducted in this laboratory.[4,25] Similarly, in this study, strains Y–H and C–164 are antigenically dissimilar although they are indistinguishable on the basis of cross-agglutination reactions. Thus, although we have assumed, for practical purposes, that all strains are antigenically homologous if they demonstrate uniform cross-agglutination reactions, this may not always be the case. Definitive identification of a leptospiral isolate can only be ascertained by agglutinin-absorption tests.

The pathogenic leptospiras have been classified into "serogroups" and "serotypes" by Wolff and Broom [11] on the basis of specific antigenic characteristics. By definition, a serotype includes all strains indistinguishable one from the other on the basis of cross-absorption procedures. Serotypes sharing major antigenic components are grouped into serogroups. Within a serogroup, in addition to antigenically heterologous serotypes, "complete" and "incomplete biotypes" may be present. In

their classification scheme, which was proposed in 1953 [26] and 1954,[11] Wolff and Broom recognized 34 serotypes which were assembled into 20 serogroups.

In this laboratory, the criteria of Wolff and Broom for the designation of serotypes have been adopted and modified to conform to our dilution techniques.[25] Thus, two strains are considered heterologous if, after absorption by the other strain, the antiserum of each strain retains at least one sixteenth of the original homologous titer. Similarly, two strains are homologous if residual titers following absorption are less than one sixteenth of the original homologous titers. Applying these criteria to the results of agglutinin-absorption tests shown (table 3), strain C–B is a complete biotype and strains S–91 and C–164 are heterologous serotypes of the type-strain Pomona. However, compared to serotypes, other than the type strain, different inter-relationships are defined under the same criteria. Thus, strain S–91, a heterologous serotype of strain Pomona, is a homologous serotype of strain V–42, which in turn is serologically identical to strain Pomona. This obvious limitation of the currently proposed classification scheme reflects the application of an arbitrary numerical basis for the designation of serotypes. In addition, residual titers of absorbed serums may not be reproducible quantitatively because of variable factors in microagglutination procedures, such as test antigen sensitivity, age, and density of test antigens.[27, 28] However, the employment of arbitrary numerical standards for serotype designations can not be discarded since the other alternative for a firm basis of antigenic classification—factor analysis—is precluded by the relatively low yield and slow growth of leptospiras in cultures.

The suggested scheme of Wolff and Broom to simplify the classification of leptospiras was an important step in that direction. However, this scheme was proposed as an interim expedient and the authors recognized that the definition of serotype and serogroup might be amended in the light of new information. Since the publication of this classification scheme, the number of serotypes has virtually doubled and there has been an increasing disclosure of new antigenic types.[4, 10, 14, 15, 29, 35] The concept of serotype and serogroup must necessarily be broadened to avoid confusion in nomenclature. This subject will be treated in an extensive study of Malayan leptospiral serotypes [14] and for this reason we do not propose at this time to apply new serotype designations for the antigenically diverse serotypes of the *pomona* group. Under a broader definition of serotype, the strains studied may not be sufficiently disparate antigenically to warrant separate appellations.

Antigenic differences among strains may be important in so far as they may be related to differing epidemiological aspects and clinical manifestations of human and animal *L. pomona* leptospirosis. In addition, diverse antigenicity in strains may explain, in part, the different cross-agglutination reactions of infected hosts in various epidemics. Coagglutinins against *Leptospira icterohaemorrhagiae*,[31] *L. sejroe*,[18] and *L. autumnalis* [4, 13] that frequently show higher titers than the homologous antibodies have been noted in mammalian hosts in various outbreaks of leptospirosis caused by strains of *L. pomona*. Finally, the antigenic attributes of strains are important in the preparation of vaccines. Additional studies on the relationship of antigenic characteristics of strains to virulence, clinical manifestation, host distribution, and host-serological response are desirable.

Summary

A serological study on 23 strains of *Leptospira pomona* is reported. The serological screening of strains employed as antigens against a battery of serotype rabbit antiserums revealed minor differences in cross-agglutination reactions. Based on these differences, strains were selected for agglutinin-absorption studies. These studies disclosed the presence of four antigenically diverse serotypes. The antigenic differences in strains may have an important bearing in epidemiological, chemical, serological, and vaccine prophylaxis aspects of *L. pomona* leptospirosis.

References

[1] Clayton, G. E. B., and Derrick, E. H.: The Presence of a Mild Type (Seven-Day Fever) in Queensland. M. J. Australia, 1. (1937): 647–664.

[2] Gsell, O.: Leptospirosen. Verlag Hans Huber, Bern, 1952.

[3] Mochtar, A.: Hundenleptospirosen in den Tropen. Japan. J. Vet. Sci., 5. (1943): 161–170.

[4] Division of Veterinary Medicine, Walter Reed Army Institute of Research, Washington, D.C.: Unpublished data.

[5] de Azevado, J. F., Valente, J. S., and Queiros, J. J. de S.: Novos Dados sobre as Leptospiras de Portugal. An. Inst. Med. Trop., *8*, (1951): 621–627.

[6] Babudieri, B., and Bianchi, L.: Untersuchungen uber ein epidemisches Vorkommen der Reisfelderleptospirose in der Provinz Pavia. Ztschr. f. Immunitätsforsch. u. exper. Therap., *98*, (1940): 37–75.

[7] Borg-Petersen, C., and Fennestad, K. L.: A Field Rodent *(Apodemus Agrarius)* as Carrier of *Leptospira Pomona* in Denmark. J.A.V.M.A., *128*, (1956): 204–205.

[8] Szeness, L., and Biro, Z.: Foci of Leptospirosis in Eastern Hungary. (In German) Acta Microbiol. Hung., *3*, (1955): 19–26 (abstr. in Vet. Bull., *266*, [1956]: 1550).

[9] Hoeden, J. van der: Epizootiology of Leptospirosis *(Canicola)* in the Bovine and Other Species in Israel. J.A.V.M.A., *126*, (1955): 207–210.

[10] Galton, M., Communicable Disease Center, Public Health Service, Chamblee, Ga.: Personal communication, 1956.

[11] Wolff, J. W., and Broom, J. C.: The Genus Leptospira Noguchi, 1917. Problems of Classification and a Suggested System Based on Antigenic Analysis. Documenta Med. Geograph. et Trop., *4*, (1954): 78–95.

[12] Gochenour, W. S., Jr., Yager, R. H., and Wetmore, P. W.: Antigenic Similarity of Bovine Strains of Leptospira (U.S.) and *Leptospira Pomona*. Proc. Soc. Exptl. Biol. and Med., *74*, (1950): 199–203.

[13] Wiesmann, E.: Die Leptospiren unter besonderer Berucksichtigung ihrer antigenen Eigenschaften. Ergebn. d. Hyg., Bakt., Immunitätsforsch. u. exper. Therap., *27*, (1952): 323–419.

[14] Alexander, A. D., Toussaint, A., Evans, L. B., Marchwicki, R., and McCrumb, F. R., Jr.: Leptospirosis in Malaya. II. Classification. In preparation.

[15] Alexander, A. D., Wetmore, P. W., Evans, L. B., Jeffries, H., and Gleiser, C. A.: Classification of Leptospiral Isolates from Malaya, Thailand and North Borneo. Am. J. Trop. Med. and Hyg., *4*, (1955): 492–506.

[16] Gochenour, W. S., Jr., Johnston, R. V., Yager, R. H., and Gochenour, W. S.: Porcine Leptospirosis. Am. J. Vet. Res., *13*, (1952): 158–160.

[17] Roberts, S. J., York, C. J., and Robinson, J. W.: An Outbreak of Leptospirosis in Horses on a Small Farm. J.A.V.M.A., *121*, (1952): 237–242.

[18] Galton, M. M., Acree, J. A., Lewis, A., and Prather, E. C.: Leptospirosis in Domestic Animals in Florida with Reference to Cattle. J.A.V.M.A., *128*, (1956): 87–91.

[19] Baker, J. A., and Little, R. B.: Leptospirosis in Cattle. J. Exptl. Med., *88*, (1948): 295–308.

[20] Sippel, W. L., Boyer, C. I., and Chambers, E. E.: Bovine Leptospirosis in Georgia. J.A.V.M.A., *120*, (1952): 277–282.

[21] Reinhard, K. R., Tierney, W. F., and Roberts, S. J.: A Study of Two Enzootic Occurrences of Bovine Leptospirosis. Cornell Vet., *40*, (1950): 148–164.

[22] Bryan, H. W., Rhoades, H. E., and Willigan, D. A.: Studies on Leptospirosis in Domestic Animals. II. Isolation of *Leptospira Pomona* from Aborted Swine Fetuses. Vet. Med., *48*, (1953): 438–439.

[23] Savino, E., and Rennella, E.: Estudios sobre Leptospiras. VI. *Leptospira suis* (n.sp.!) y *Leptospira hyos* (n. sp.!) Aislaida en Cerdos de la Republica Argentina. Rev. inst. bacteriol. Malbrun, *13*, (1945–1948): 62–65.

[24] Gochenour, W. S., Jr., and Yager, R. H.: Classification of North American Leptospiras. Proc. Book, AVMA (1952): 178–183.

[25] Alexander, A., Evans, L. B., Jeffries, H., Gleiser, C., and Yager, R. H.: Serologic Characterization of the Fort Bragg Leptospire. Proc. Soc. Exptl. Biol. and Med., *86*, (1954): 405–408.

[26] Wolff, J. W.: The Classification of Pathogenic Leptospires. Symposium on the Leptospiroses. M. Sci. Publ., *1*, (1953): 174–182.

[27] Borg-Petersen, C., and Fagraes, A.: The Influence of the Antigen Density and Other Factors on the Serum Titer in the Agglutination-Lysis Test for Leptospirosis. Acta Path. et Microbiol. Scandinav., *26*, (1949): 555–567.

[28] Stoenner, H. G.: Application of Serology to the Diagnosis of Leptospirosis. Proc. Book, AVMA (1955): 172–176.

[29] Riel, J. van, Szpajshendler, L., and Riel, M. van: Etude Clinique, Bacteriologique et Epidemiologique d'un Noveau Foyer de Leptospirose au Congo Belge. Bull. Soc. path. exot., *49*, (1956): 118–143.

[30] Smith, D. J. W., Brown, H. E., Tonge, J. I., Sinnamon, C. N., Macdonald, V. M., Ross, C. J., and Doherty, R. L.: The Serological Classification of 89 Strains of Leptospirae from North Queensland, Including Five Serotypes New to Australia. Austral. Ann. Med., *3*, (1954): 98–105.

[31] Morse, E. V., and Allen, V.: Serological Cross-Agglutination Reactions Between *Leptospira Pomona* and *Leptospira Icterohaemorrhagiae*, AB. Am. J. Vet. Res., *17*, (1956): 563–568.

COPYRIGHT 1957 BY AMERICAN VETERINARY MEDICAL ASSOCIATION

Printed in the U.S.A.